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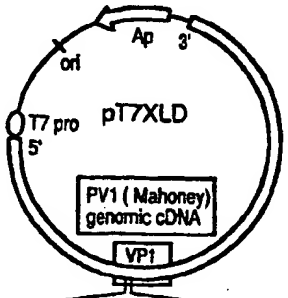

Sal I Hind III

nt2753 G|TC GAC AAC CCA GCT TCC ACC ACC ATT AAG GAC A|AG CTT 2791
CAG CT|G TTG GGT CGA AGG TGG TGG TTA TTC CTG TTC GA|A
aa1092 V D N P A S T T N K D K L 1104

Clone	Sequence encoded (Chlamydial sequences underlined)	Viable virus recovered/ strain designation
pT7XLD	NPASTT NKD	Yes PV1-XLD
pT7Ct2	VAGLEKDPVA	No
pT7Ct4	N PTTSDVAGLEKDPVA	No
pT7Ct5	N PTTSDVAGLEKDPKD	Yes PV1-Ct5
pT7Ct7	NPASTTSDVAGLEKDPVA	Yes PV1-Ct7
pT7Ct8	NPASTTSDVAGLEKDPKD	Yes PV1-Ct8

(57) Abstract

Hybrid picornaviruses expressing chlamydial epitopes from the major outer membrane protein of *Chlamydia trachomatis* in a functional form are described. The hybrid viruses grow to high titre in cell culture and when administered to mammals induce an immune response against both the picornavirus and *C. trachomatis*. The antisera from immunised mammals neutralised both homotypic and heterotypic serovars of *C. trachomatis*. The hybrid picornaviruses have utility as vaccines and as tools for the generation of immunological reagents. Methods for modifying surface exposed loops of known sequences to produce hybrid proteins are described.

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TITLE OF THE INVENTION

HYBRID PICORNAVIRUSES EXPRESSING CHLAMYDIAL EPITOPES

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of United States patent application Serial No. 060,978 filed May 13, 1993.

FIELD OF THE INVENTION

The invention relates to the field of immunology and in particular relates to hybrid picornaviruses expressing at least one chlamydial epitope.

BACKGROUND OF THE INVENTION

Chlamydia trachomatis is a species of the genus Chlamydiaceae, order Chlamydiales. C.trachomatis infects the epithelia of the conjunctivae and the genital tract, causing trachoma and a variety of sexually transmitted diseases (STDs) which can lead to, respectively, blindness or infertility. There are at least 15 serovars of C.trachomatis, of which A, B, and C are causative agents of trachoma, while serovars D, E, F, G, H, I, J, and K are the most common causative agents of chlamydial STDs. C.trachomatis infections are endemic throughout the world. Trachoma is the leading cause of preventable blindness in developing nations, and it is estimated that 600 million people suffer from trachoma worldwide, with as many as 10 million of them being blinded by the disease. In the United States there are an estimated 3 million cases per year of STDs caused by C.trachomatis. An effective vaccine would be useful in the control of chlamydial disease (ref. 39 - the references are listed at the end of the description).

The pathogenesis of trachoma involves repeated ocular infections and the generation of a deleterious hypersensitivity response to chlamydial antigen(s) (refs. 15, 16, 35). The available evidence supports the hypothesis that secretory IgA is an important component of protection. Ocular infection in a primate model

induces rapid and persistent production of IgA in tears, whereas the presence of IgG in tears is transient, corresponding to the period of peak conjunctival inflammation (ref. 7). Protective immunity following experimental ocular infection in a sub-human primate model is homotypic and resistance to ocular challenge correlates with the presence of serovar-specific antibodies in tears (refs. 15, 16, 38). Tears from infected humans neutralised the infectivity of homologous but not heterologous trachoma serovars for owl monkey eyes (ref. 29) whereas passive humoral immunization with anti-trachoma antibodies was not protective (ref. 30). Past attempts to develop whole-cell vaccines against trachoma have actually potentiated disease by sensitizing vaccinees (refs. 15, 16). Sensitization has been determined to be elicited by a 57kD stress response protein (SRP) present in all serovars of C.trachomatis. Repeated exposure to the 57kD SRP can result in a delayed hypersensitivity reaction, causing the chronic inflammation commonly associated with chlamydial infections (refs. 39, 40). Thus an immunogenic preparation capable of inducing a strong and enduring mucosal neutralising antibody response including a mucosal response without sensitizing the vaccinee would be useful.

A promising candidate antigen for the development of an immunogenic composition useful as a vaccine is the chlamydial major outer membrane protein (MOMP) (refs. 6, 34). Other surface proteins and the surface lipopolysaccharide are also immunogenic, but the antibodies they induce have not been found to be protective (refs. 42, 43). The MOMP, which is the predominant surface protein, is an integral membrane protein with a mass of about 40kDa. which, with the exception of four variable domains (VDs) designated I, II, III, and IV, is highly conserved amongst serovars.

The sequences of all four VDs have been determined for fifteen serovars (refs. 12, 41). Antibodies capable of neutralising chlamydial infectivity recognise the MOMP (refs. 21, 31, 33, 34, 42, 43). Epitopes to which MOMP-specific neutralising monoclonal antibodies bind have been mapped for serovars A, B and C (refs. 3, 8, 32, 42, 43), and may represent appropriate targets for the development of synthetic or subunit vaccines. The binding sites are contiguous sequences of six to eight amino acids located within VDs I or II, and IV, depending on the serovar. Subunit immunogens (e.g. isolated MOMP or synthetic peptides) expressing MOMP epitopes can induce antibodies capable of recognising intact chlamydiae (refs. 9, 33). However, conventionally administered subunit immunogens are generally poor inducers of mucosal immunity. It would be useful to provide chlamydial epitopes in such a way as to enhance their immunogenicity.

Poliovirus is an enterovirus, a genus of the family Picornaviridae. There are three distinct serotypes of the virus, and multiple strains within each serotype. Virulent strains are causative agents of paralytic poliomyelitis. Attenuated strains, which have reduced potential to cause paralytic disease, and inactivated virulent strains are used as vaccines. Infection with the virus induces long-lasting, protective, mucosal immunity. Inoculation with inactivated poliovirus vaccines can also induce a mucosal immune response.

The structure of poliovirus is known, and is highly conserved among strains and serotypes. The structures of several other picornaviruses (viruses belonging to genera of the family Picornaviridae) have also been determined, and have been shown to be closely related to the structure of poliovirus. It is possible to express foreign epitopes on the capsid of polioviruses (refs. 1, 4, 5, 11, 14, 18, 19, 20, 22, 23, 24, 25, 26, 27, 28) and

this work has been extended to other picornaviruses (ref. 2). As might be expected from their closely related structures, similar results have been obtained with other picornaviruses. Epitopes which have been expressed are usually short, well defined, contiguous epitopes, and most have been expressed within poliovirus neutralisation antigenic site I (NAgI) or the equivalent site on other picornaviruses. This site includes the loop linking beta strands B and C (the BC loop) of poliovirus capsid protein VP1. The BC loop of VP1 is a surface-exposed loop of nine amino acids (ref. 17) which can be replaced and extended with at least twenty-five heterologous amino acids (ref. 26). It is also possible to express epitopes at other sites on the surface of picornaviruses, for example within other neutralisation antigenic sites of poliovirus (refs. 22, 27).

Despite the examples of picornavirus hybrids described in the literature, the factors governing success or failure in the construction of particular hybrids remain unclear. Frequent problems are failure to recover viable virus (ref. 11) and poor immunogenicity (refs. 11, 20). The volume and the net charge of hybrid BC loops have been proposed as factors influencing the viability of poliovirus hybrids, but in both cases any influence has yet to be conclusively demonstrated (refs. 10, 11). Retaining at least a part of the native BC loop sequence has also been suggested to favourably influence the growth characteristics of hybrid polioviruses, but which parts of the sequence to retain were not clearly defined (ref. 26). In addition, many viable hybrids which express a foreign epitope are unable to induce a strong immune response capable of recognising the foreign epitope in its normal context. There is some data which suggests that the length of the hybrid BC loop may influence its antigenicity, in that a longer than normal loop might be more flexible and hence better able to

adopt an appropriate conformation, but the precise effects of any changes in length are unknown (ref. 26). Hybrid polioviruses expressing chlamydial epitopes, which grow to a high titre and are immunogenic, would be useful as vaccines and as tools for the generation of immunological reagents.

Previously described poliovirus hybrids expressing chlamydial amino acid sequences were able to induce an immune response which recognised chlamydial MOMP, but only the MOMP of one or possibly two serovars (ref. 23). Because there are at least fifteen chlamydial serovars it would be useful for a hybrid expressing a chlamydial epitope to induce an immune response which can recognise, and neutralise, several serovars.

C.trachomatis naturally infects the mucosal surfaces of the eye and genital tract, and secretory IgA is probably an important component of protection (refs. 7, 15, 16, 29, 30, 39). Consequently, it would be useful for a chlamydial vaccine to induce a mucosal immune response. Since poliovirus infection efficiently produces a mucosal immune response, the poliovirus hybrids expressing a chlamydial epitope should be useful for targetting the production of anti-chlamydial antibodies to mucosal surfaces of the eye and genital tract. Furthermore, because the hybrids do not express sequences from the 57kD SRP, they should be useful for inducing an anti-chlamydial immune response without the disadvantageous risk of potentiating disease by sensitizing the vaccinee.

SUMMARY OF INVENTION

The present invention is directed towards the provision of improved hybrid picornaviruses expressing chlamydial epitopes.

According to one aspect of the present invention, there is provided a hybrid picornavirus expressing at least one chlamydial epitope and capable of inducing

antibodies immuno-reactive with at least three different Chlamydia serovars. At least three different Chlamydia serovars are conveniently three different Chlamydia trachomatis serovars and the hybrid picornavirus may be a hybrid poliovirus, including an attenuated poliovirus.

The at least one chlamydial epitope may comprise a linear amino acid sequence of between about 5 and about 15 amino acids, comprising an epitope of the major outer membrane protein of Chlamydia trachomatis. The chlamydial epitope may comprise a serovar-specific epitope included in, for example, a variable domain I sequence, including an amino acid sequence selected from the group consisting of VAGLEK (SEQ. ID NO: 1), PTTSDVAGLEKDP (SEQ. ID NO: 24), TTSDVAGLEKDPVA (SEQ. ID NO: 26) and TTSDVAGLEKDP (SEQ. ID NO: 10). The chlamydial epitope may also comprise a species-specific epitope included in, for example, the variable domain IV sequence, including an amino acid sequence selected from the group consisting of LNPTIAG (SEQ. ID NO: 9), TTLNPTIAGAGDVK (SEQ. ID NO: 11), TTLNPTIAGAGD (SEQ. ID NO: 12) and TTLNPTIAGA (SEQ. ID NO: 13). In an embodiment of the present invention, the hybrid poliovirus is capable of growing to at least about 1%, and preferably at least about 10% and most preferably at least about 50% of the titre of non-hybrid poliovirus grown under the same conditions.

In a further embodiment of the present invention, at least one chlamydial epitope is contained within a hybrid BC loop sequence of poliovirus capsid protein VP1 or other hybrid loop sequence of a poliovirus capsid protein, in particular a BC loop sequence extended by the chlamydial epitope, which may be a serovar-specific epitope. As noted above, the serovar-specific epitope of the major outer membrane protein may comprise a variable domain I sequence, including one of the specific amino acid sequences clearly described. The chlamydial epitope

may be contained within a hybrid BC loop having, for example, an amino acid sequence selected from the group consisting of ASTTSDVAGLEKDPVAKL (SEQ. ID NO: 15) and ASTTSDVAGLEKDPKDKL (SEQ. ID NO: 16).

The chlamydial epitope may also comprise a species-specific epitope contained within a hybrid BC loop. As noted above, the species-specific epitope may comprise a variable domain IV sequence, including one of the specific amino acid sequences clearly described. The chlamydial epitope may be contained in a hybrid BC loop having, for example, the amino acid sequence selected from the group consisting of ASTTLNPTIAGAGDVKDKL (SEQ. ID NO: 17), ASTTLNPTIAGAGDDKL (SEQ. ID NO: 18) and ASTTLNPTIAGADKL (SEQ. ID NO: 19). The present invention also includes functional variants of the elements of the hybrid sequence. Examples of such functional variants and their prediction is set forth in Table 5 below (the Tables appear at the end of the disclosure).

The present invention, in accordance with another aspect, provides an isolated nucleic acid molecule comprising at least a portion encoding the hybrid picornavirus provided herein and described above.

In addition, in accordance with a further aspect, the present invention provides an immunogenic composition comprising an immunoeffective amount of a hybrid picornavirus or an isolated nucleic acid molecule as provided herein. Such immunogenic composition may be formulated for mucosal or parenteral administration. Such immunogenic composition may comprise at least one other immunogenic or immunostimulating material, such as an adjuvant, which may be aluminum phosphate or aluminum hydroxide.

Such immunogenic composition may be formulated as a vaccine for human administration wherein the hybrid picornavirus is attenuated or inactivated, or the

isolated DNA molecule codes for an attenuated hybrid picornavirus.

In an additional aspect, the present invention provides a method of immunizing a host, which may be a human, which comprises administering to the host, the immunogenic composition provided herein. In the case of administration to a human host, the hybrid picornavirus is attenuated or inactivated, or the DNA codes for an attenuated strain of the hybrid picornavirus.

Further, the present invention includes, in a yet further aspect thereof, a method of determining the presence of chlamydia in a sample, which comprises the steps of (a) immunizing a host with an immunogenic composition provided herein to produce a chlamydia-specific antibodies, (b) contacting the sample with the chlamydia-specific antibodies to produce complexes comprising the chlamydia-specific antibodies and chlamydia, and (c) determining the production of the complexes. The invention further includes a chlamydia-specific antibody which is immunoreactive with at least three different Chlamydia serovars producible by immunizing a host with the immunogenic composition provided herein. This antibody may be used in a diagnostic kit for detecting the presence of chlamydia in a sample along with means for contacting the antibody with the sample to produce a complex comprising chlamydia and chlamydia-specific antibody and means for determining production of the complex.

In this application, a hybrid BC loop includes a BC loop into which has been inserted a chlamydial epitope, which insertion may have involved deletion and/or replacement of native BC loop amino acids.

The present invention further includes procedures for modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein. The protein may be a viral protein, particularly a picornavirus.

Accordingly, in an additional aspect of the present invention, there is provided a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises (a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility; (b) providing a first nucleic acid molecule encoding at least the protein; (c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response; (d) identifying a first amino acid proximate the amino terminus of the target sequence which is the same as or functionally equivalent to a second amino acid in the identified portion; (e) excising between one and all codons encoding said identified portion from the first nucleic acid molecule to produce an excised sequence the 5′ codon of which encodes the second amino acid, an upstream codon which is the codon immediately preceding the excised sequence, and a downstream codon which is the codon immediately following the excised sequence; (f) deleting zero or at least one codon from the 5′ end of the second nucleic acid molecule to produce a third nucleic acid molecule the 5′ codon of which is the codon encoding the first amino acid; (g) inserting the third nucleic acid molecule between the upstream and downstream codons such that the 5′ codon of the inserted third nucleic acid molecule encodes the first amino acid to produce a fourth nucleic acid molecule encoding the hybrid protein; and (h) producing the hybrid protein from the fourth nucleic acid molecule.

In a further aspect of the present invention, there is provided a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises (a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility; (b) providing a

first nucleic acid molecule encoding at least the protein; (c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response; (d) identifying a first amino acid proximate the amino terminus of the target sequence which is the same as or functionally equivalent to a second amino acid in the identified portion; (e) deleting the codon encoding the first amino acid and all preceding codons from the 5' end of the second nucleic acid molecule to produce a third nucleic acid molecule; (f) identifying in the first nucleic acid molecule an upstream codon which is the codon encoding the second amino acid and a downstream codon which is the immediately adjacent codon at the 3' end of the upstream codon; (g) forming an insertion site in the first nucleic acid molecule between the upstream and the downstream codon; (h) inserting the third nucleic acid molecule into the first nucleic acid molecule at the insertion site such that the 5' codon of the inserted third nucleic acid molecule encodes the first amino acid, to produce a fourth nucleic acid molecule encoding the hybrid protein; and (i) producing the hybrid protein from the fourth nucleic acid molecule.

An additional aspect of the present invention provides a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises (a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility; (b) providing a first nucleic acid molecule encoding at least the protein; (c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response; (d) identifying a first amino acid proximate the carboxy terminus of the target sequence which is the same as or functionally equivalent to a second amino acid in the identified portion; (e) excising between one and

all codons encoding the identified portion from the first nucleic acid molecule to produce an excised sequence the 3' codon of which encodes the second amino acid, an upstream codon which is the codon immediately preceding the excised sequence, and a downstream codon which is the codon immediately following the excised sequence; (f) deleting zero or at least one codon from the 3' end of the second nucleic acid molecule to produce a third nucleic acid molecule the 3' codon of which is the codon encoding the first amino acid; (g) inserting the third nucleic acid molecule between the upstream and downstream codons such that the 3' codon of the inserted third nucleic acid molecule encodes the first amino acid to produce a fourth nucleic acid molecule encoding the hybrid protein; and (h) producing the hybrid protein from the fourth nucleic acid molecule.

A further aspect of the present invention provides a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises (a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility; (b) providing a first nucleic acid molecule encoding at least the protein; (c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response; (d) identifying a first amino acid proximate the carboxy terminus of the target sequence which is the same as or functionally equivalent to a second amino acid in the identified portion; (e) deleting the codon encoding the first amino acid and all following codons from the 3' end of the second nucleic acid molecule to produce a third nucleic acid molecule; (f) identifying in the first nucleic acid molecule a downstream codon which is the codon encoding the second amino acid and an upstream codon which is the immediately adjacent codon at the 5' end of the downstream codon; (g) forming an insertion

site in the first nucleic acid molecule such that the codon directly following the insertion site encodes the second amino acid; (h) inserting the third nucleic acid molecule into the first nucleic acid molecule at the insertion site such that the 3' codon of the inserted third nucleic acid molecule encodes the first amino acid, to produce a fourth nucleic acid molecule encoding the hybrid protein; and (i) producing the hybrid protein from the fourth nucleic acid molecule.

Another further aspect of the present invention, there is provided a method of modifying a protein having a surface exposed loop of known sequence, wherein the first nucleic acid molecule comprises a first nucleic acid cassette including the upstream codon and the downstream codon, which method comprises (a) providing a second nucleic acid cassette having an upstream sequence, a downstream sequence, and a sequence between the upstream and the downstream sequence having the same sequence as the third nucleic acid molecule, such that the upstream sequence encodes the same amino acid sequence as the sequence of the first cassette from its 5' end to the upstream codon inclusive and the downstream sequence encodes the same amino acid sequence as the sequence of the first cassette from the downstream codon to its 3' end inclusive; (b) excising the first nucleic acid cassette from the first nucleic acid molecule to form a fifth nucleic acid molecule; (c) inserting the second nucleic acid cassette into the second nucleic acid molecule to form a sixth nucleic acid molecule; and (d) producing the hybrid protein from the sixth nucleic acid molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the strategy for construction of hybrid poliovirus expressing at least one chlamydial immunostimulating epitope using a SalI-HindIII

mutagenesis cartridge according to one embodiment of the present invention;

Figure 2 shows the single-step growth curves of hybrid poliovirus expressing at least one chlamydial immunostimulating epitope according to one embodiment of the present invention;

Figure 3 shows the specificity of rabbit anti-PV1-Ct7 and anti-PV1-Ct8 antisera for MOMP from C.trachomatis serovars A, B and C by Western blot analysis;

Figure 4 shows the specificities of rabbit anti-PV1-Ct7 (#13 to #16), and anti-PV1-Ct8 (#17 to #20) antisera by dot-immunoblot to different C.trachomatis serovars;

Figure 5 shows neutralisation of C.trachomatis serovars A (left hand panel) and C (right hand panel) by anti-PV1-Ct7 rabbit antisera;

Figure 6 shows the strategy for construction of hybrid poliovirus expressing at least one chlamydial immunostimulating epitope using a SalI - Hind III mutagenesis cartridge according to another embodiment of the present invention;

Figure 7 shows the single-step growth curves of further hybrid poliovirus expressing at least one chlamydial immunostimulating epitope according to another embodiment of the present invention;

Figure 8 shows the specificity of pooled rabbit anti-PV1-CtIVA (A), anti-PV1-CtIVB (B), anti-PV1-CtIVC (C), and anti PV1-XLD (D) antisera for MOMP from C.trachomatis serovars by Western blot analysis;

Figure 9 shows the specificities of rabbit anti-PV1-CtIVA (#24 to 26), anti-PV1-CtIVB (#27 to 29) and anti-PV1-CtIVC (#30 to #32) antisera by dot-immunoblot to fifteen C.trachomatis serovars and to the guinea pig inclusion conjunctivitis (GPIC) strain of C.psittaci;

Figure 10 shows neutralisation of C.trachomatis serovars by anti-PV1-CtIVA, anti-PV1-CtIVB and anti-PV1-CtIVC rabbit antisera;

Figure 11 contains a schematic diagram of a method of modifying a protein according to one embodiment of the invention;

Figure 12 contains a schematic diagram of a method of modifying a protein according to a further embodiment of the invention;

Figure 13 contains a schematic diagram of a method of modifying a protein according to a yet further embodiment of the invention;

Figure 14 contains a schematic diagram of a method of modifying a protein according to a yet additional embodiment of the invention; and

Figure 15 contains a schematic diagram of a method of modifying a protein according to a yet further embodiment of the invention.

GENERAL DESCRIPTION OF INVENTION

Referring to Figure 1, there is illustrated a strategy for construction of polio/chlamydia serovar A hybrids according to an embodiment of the present invention. Thus, using a SalI - Hind III mutagenesis cartridge, the PV1-Mahoney cDNA clone pT7XLD was modified to encode amino acid sequences, including the VAGLEK epitope (SEQ. ID NO: 1), from C.trachomatis serovar A MOMP VDI. The mutagenesis cartridge is contained between poliovirus nucleotides 2753 to 2791 (SEQ. ID NO: 32), which encode poliovirus amino acids 1092 to 1104 (SEQ. ID NO: 31) as shown in Figure 1. These amino acids include the BC loop of VP1, which has the sequence ASTTNKDKL (SEQ.ID NO: 14) and is underlined in Figure 1. In this application, we use the four-digit code for poliovirus amino-acids; for example, 1095 is amino acid 95 of capsid protein VP1. The polio-specific nucleotide sequence within the cartridge was replaced with synthetic

oligonucleotides encoding the following amino acid sequences from C.trachomatis serovar A MOMP VDI:

Clone	Sequence encoded (Chlamydial sequences underlined)	
pT7XLD	NPASTT NKD	(SEQ. ID NO: 20)
pT7Ct2	VAGLEKDPVA	(SEQ. ID NO: 21)
pT7Ct4	N PTTSDVAGLEKDPVA	(SEQ. ID NO: 5)
pT7Ct5	N PTTSDVAGLEKDPKD	(SEQ. ID NO: 6)
pT7ct7	NPASTTSDVAGLEKDPVA	(SEQ. ID NO: 7)
pT7Ct8	NPASTTSDVAGLEKDPKD	(SEQ. ID NO: 8)

These sequences are indicated in Figure 1. RNA transcripts were prepared from these constructs and used to transfect Vero cells to produce viable hybrid polioviruses PV1-Ct5, PV1-Ct7 and PV1-Ct8 expressing chlamydial immunostimulating epitopes as shown in Figure 1.

Figure 2 illustrates the single-step growth curves of PV1-XLD, PV1-Ct7 and PV1-Ct8, and as shown in Table 1 both viruses PV1-Ct7 and PV1-Ct8 were neutralised by monoclonal antibody A-20 which is specific for the VAGLEK (SEQ. ID NO: 1) epitope from VDI of C.trachomatis. Thus, hybrid poliovirus expressing chlamydial immunostimulating epitopes that grow to a high titre are provided in accordance with one embodiment of the present invention.

When used to immunize rabbits, the hybrid poliovirus induced high titre antisera against C.trachomatis. Figure 3 shows the specificity of rabbit anti-PV1-Ct7 and anti-PV1-Ct8 antisera for MOMP from C.trachomatis serovars A, B and C by Western blot analysis.

Figure 4 shows the test results of a dot blot assay to determine the reactivity of the antisera produced by immunization with PV1-Ct7 and PV1-Ct8 with MOMP in the context of intact chlamydial elementary bodies. These results indicated that hybrid polioviruses expressing chlamydial immunostimulating epitopes that grow to a high titre and are capable of inducing antibodies cross-

reactive with at least three C.trachomatis serovars are provided in accordance with one embodiment of the present invention.

Figure 5 shows neutralisation of C.trachomatis serovars A (left hand panel) and C (right hand panel) by anti-PV1-Ct7 antisera from rabbits 13 to 16. Pooled anti-PV1-XLD antisera was non-neutralising (not shown). These results indicated that hybrid poliovirus expressing chlamydial immunostimulating epitopes that grow to a high titre are capable of inducing antibodies cross reactive with at least three C.trachomatis serovers, and that the antibodies can neutralize (or kill) live C.trachomatis in accordance with an embodiment of the present invention. In accordance with one aspect of the invention there is provided immunogenic compositions comprising the hybrid poliovirus expressing immunostimulating epitopes. One utility of such immunogenic compositions is as vaccines against C.trachomatis and it is desirable that antibodies produced by the administration of such immunogenic compositions should neutralize C.trachomatis. Another utility of such immunogenic compositions is to produce antibodies against C.trachomatis for use as an immunotherapeutic agent or an immunodiagnostic agent.

Referring to Figure 6, there is illustrated a strategy for construction of polio/chlamydia serovar D hybrids according to an embodiment of the present invention. Thus, using a SalI - Hind III mutagenesis cartridge, the PV1-Mahoney cDNA clone pT7XLD was modified to encode amino acid sequences, including the LNPTIAG (SEQ. ID NO: 9) epitope, from C.trachomatis serovar D MOMP VDIV. The mutagenesis cartridge is contained between poliovirus nucleotides 2753 to 2791 (SEQ. ID NO: 32), which encode poliovirus amino acids 1092 to 1104 (SEQ. ID NO: 31) as shown in Figure 6. These amino acids include the BC loop of VP1, which has the sequence ASTTNKDKL (SEQ. ID NO: 14) and is underlined in Figure 6.

In this application, we use the four-digit code for poliovirus amino-acids; for example, 1095 is amino acid 95 of capsid protein VP1. The polio-specific nucleotide sequence within the cartridge was replaced with synthetic oligonucleotides encoding the following amino acid sequences from C.trachomatis serovar D MOMP VDIV:

Clone	Sequence encoded (Chlamydial sequences underlined)	
pT7XLD	NPASTT NKD	(SEQ. ID NO: 20)
pT7CtIVA1	NPASTTLNPTIAGAGDVKD	(SEQ. ID NO: 2)
pT7CtIVB4	NPASTTLNPTIAGAGD D	(SEQ. ID NO: 3)
pT7CtIVC9	NPASTTLNPTIAGA D	(SEQ. ID NO: 4)

These sequences are indicated in Figure 6. RNA transcripts were prepared from these constructs and used to transfect Vero cells to produce viable hybrid polioviruses PV1-CtIVA, PV1-CtIVB and PV1-CtIVC expressing chlamydial immunostimulating epitopes as shown in Figure 6.

Figure 7 illustrates the single-step growth curves of PV1-XLD, PV1-CtIVA, PV1-CtIVB and PV1-CtIVC and demonstrates that further hybrid polioviruses expressing chlamydial immunostimulating epitopes that grow to a high titre are provided in accordance with an embodiment of the present invention.

When used to immunize rabbits, the hybrid polioviruses PV1-CtIVA, PV1-CtIVB and PV1-CtIVC induced high titre antisera against C.trachomatis and Figure 8 shows the specificity of pooled rabbit anti-PV1-CtIVA (A), anti-PV1-CtIVB (B), anti-PV1-CtIVC (C), and anti-PV1-XLD (D) antisera for MOMP from 15 C.trachomatis serovars by Western blot analysis.

Figure 9 shows the test results of a dot blot assay to determine the reactivity of the anti-sera produced by immunization with PV1-CtIVA, PV1-CtIVB and PV1-CtIVC with MOMP in the context of intact chlamydial elementary bodies from 15 C.trachomatis serovars.

The results shown in Figures 8 and 9 indicated that further hybrid polioviruses expressing chlamydial immunostimulating epitopes that grow to a high titre and are capable of inducing antibodies cross-reactive with at least three C.trachomatis serovars are provided in accordance with an embodiment of the present invention.

Figure 10 shows neutralisation of C.trachomatis serovars B, Ba, D, E, F, G, A, C, H, I, J, & K by pooled rabbit antisera against PV1-XLD (panel XLD), PV1-CtIVA (panel CtIV A1), PV1-CtIVB (panel CtIV B4), and PV1-CtIVC (panel CtIV C9). The bars in each panel indicate the neutralising titer of the sera on a $\log_2$ scale. These results further indicated that hybrid polioviruses expressing chlamydial immunostimulating epitopes that grow to a high titre are capable of inducing antibodies cross-reactive with at least three C.trachomatis serovars, and that the antibodies can neutralise (or kill) live C.trachomatis in accordance with an embodiment of the present invention.

Referring to Figure 1, clone pT7Ct2 replaced the native sequence NPASTTNKD (SEQ. ID NO: 20) with the VDI sequence VAGLEKDPVA (SEQ. ID NO: 21), whereas clone pT7Ct4 replaced the native PASTTNKD (SEQ. ID NO: 22) with the chlamydial sequence PTTSDVAGLEKDPVA (SEQ. ID NO: 23). Neither were viable. Clone pT7Ct5 replaced the native PASTTN (SEQ. ID NO: 30) with the chlamydial sequence PTTSDVAGLEKDP (SEQ. ID NO: 24), differing from pT7Ct4 by only two amino acids. This difference was sufficient to produce a viable virus, although the virus grew very poorly and was not characterised further. Clone pT7Ct7 replaced the native TTNKD (SEQ. ID NO: 25) with the chlamydial sequence TTSDVAGLEKDPVA (SEQ. ID NO: 26), whereas clone pT7Ct8 replaced the native TTN (SEQ. ID NO: 27) with the chlamydial sequence TTSDVAGLEKDP (SEQ. ID NO: 10), differing from pT7Ct7 by only two amino acids. These two clones were designed so that less than six

amino-acids were deleted from the native BC loop, and so that deleted native amino acids and inserted chlamydial sequences were located in the part of the BC loop displaying greatest mobility (determined on the basis of crystallographic temperature factors). The chlamydial VDI sequences were not restricted to the known VAGLEK (SEQ. ID NO: 1) epitope or to the sequence DVAGLEKD (SEQ. ID NO: 28) expressed in previously described hybrids (ref. 23), but instead included adjacent sequences from VDI. These adjacent sequences were selected to increase homology of the expressed VDI sequences with VDI sequences of other chlamydial serovars, and so that the amino-terminal amino acids were the same as the amino-terminal amino acids of the deleted BC loop amino acids. Referring to Figure 6 it can be seen that the design strategy used successfully for PV1-Ct7 and PV1-Ct8 was also used successfully to design the hybrid picornaviruses PV1-CtIVA, PV1-CtIVB and PV1-CtIVC, which expressed a different chlamydial epitope. This shows that this design strategy is generally applicable to the expression of chlamydial epitopes on hybrid polioviruses and indeed to the expression of epitopes of other proteins on picornaviruses. This procedure constitutes another aspect of the present invention and is further illustrated in Figures 11 to 15.

In a preferred embodiment of the aspects of the invention relating to protein modification, the protein is a virus protein, particularly a picornavirus protein and the hybrid protein is disposed on a hybrid picornavirus, particularly a hybrid poliovirus. As described earlier, such hybrid picornavirus may express at least one chlamydial epitope. The target sequence may elicit a B- or a T-cell immune response. The deletion and insertion steps conveniently may be effected by cassette mutagenesis and, in particular, with the excised sequence contained within a first mutagenesis cassette

and the inserted sequence contained within a second mutagenesis cassette.

The portion of the loop having the greatest mobility can be conveniently determined by NMR, crystallographic temperature factors, fluoresence anisotropy and other spectroscopic and biophysical techniques (see, for example, ref. 46). The portion having greatest mobility is typically the same as the portion with greatest solvent accessibility and a comparison to homologous proteins of known crystal structure can aid in the determination of such a portion for a particular loop to be modified in accordance with these procedures.

Referring to Figure 11, there is illustrated a flow diagram describing a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein in accordance with the first of the aspects of the present invention relating to protein modification. The method includes determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility. Then, the sequence of a target amino acid sequence intended for eliciting an immune response is compared with the amino acid sequence of the portion having greatest mobility to identify a first amino acid proximate the amino terminus of the target sequence that is the same as or functionally equivalent to a second amino acid in the identified portion. Between one and all codons encoding the identified portion is excised from a first nucleic acid molecule encoding at least the protein, to produce an upstream codon, a downstream codon and an excised sequence, the 5′ codon of which encodes the second amino acid.

Then, zero or at least one codon is deleted from the 5′ end of a second nucleic acid molecule encoding the target amino acid sequence to produce a third nucleic acid molecule, the 5′ codon of which is the codon

encoding the first amino acid. This third nucleic acid molecule is then inserted between the upstream and downstream codons, such that the 5' codon of the third nucleic acid molecule encodes the first amino acid, to produce a fourth nucleic acid molecule encoding the hybrid protein. The hybrid protein is then expressed from the fourth nucleic acid molecule in an appropriate system.

In an alternative embodiment illustrated in Figure 12 and corresponding to the second of the aspects of the invention relating to protein modification, the first and second amino acids are identified as shown in Figure 11. An insertion site is formed in the first nucleic acid molecule encoding at least the protein such that the codon directly preceding the insertion site encodes the second amino acid. The codon encoding the first amino acid and all preceding codons are then deleted from the second nucleic acid molecule encoding the target sequence to produce a third nucleic acid molecule. The third nucleic acid molecule then is inserted into the nucleic acid molecule encoding at least the protein at the insertion site such that the 5' codon of the inserted nucleic acid molecule encodes the first amino acid, to produce a fourth nucleic acid molecule encoding the hybrid protein. The hybrid protein is then expressed from the fourth nucleic acid molecule in an appropriate system.

Referring to Figure 13, which corresponds to the third of the aspects of the invention relating to protein modification, there is illustrated a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein in accordance with a further embodiment of the present invention. The method includes determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility. Then, the sequence of a target amino acid sequence

intended for eliciting an immune response is compared with the amino acid sequence of the portion having greatest mobility to identify a first amino acid proximate the carboxy terminus of the target sequence that is the same as or functionally equivalent to a second amino acid in the identified portion. Between one and all codons encoding the identified portion having the greatest mobility are then deleted from a first nucleic acid molecule encoding at least the protein to produce an upstream codon, a downstream codon and an excised sequence, the 3' codon of which encodes the second amino acid.

Then zero or at least one codon is deleted from the 3' end of a second nucleic acid molecule encoding the target amino acid sequence for eliciting an immune response, to produce a third nucleic acid molecule, the 3' codon of which encodes the first amino acid. This third nucleic acid is then inserted between the upstream and downstream codons such that the 3' codon of the inserted third nucleic acid encodes the first amino acid to produce a fourth nucleic acid molecule encoding the hybrid protein. The hybrid protein is then expressed from the fourth nucleic acid molecule in an appropriate system.

Referring to Figure 14, which corresponds to the fourth of the embodiments of the invention relating to protein modification, there is illustrated a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein in yet another embodiment of the invention. In this method, a first amino acid proximate the carboxy terminus of a target sequence for eliciting an immune response is identified as being the same or functionally equivalent to a second amino acid in a portion of the loop having greatest mobility as described above and in Figure 13. An insertion site is then formed in a first nucleic acid

molecule encoding at least the virus protein such that the codon directly following the insertion site encodes the second amino acid. The codon encoding the first amino acid and all following codons are then deleted from the second nucleic acid molecule to produce a third nucleic acid molecule. The third nucleic acid molecule is then inserted at the insertion site such that the 3' codon of the inserted third nucleic acid molecule encodes the first amino acid, to produce a fourth nucleic acid molecule encoding the hybrid protein. The hybrid protein is then expressed from the fourth nucleic acid molecule in a suitable system.

Referring to Figure 15, there is illustrated a method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein in a further embodiment of the invention. In this embodiment, the upstream and downstream codons shown in any one of Figures 11, 12, 13, or 14 are included in a first nucleic acid cassette within the DNA molecule encoding at least the protein. A second nucleic acid cassette is provided in which a sequence encoding a target amino acid sequence is located between an upstream sequence and a downstream sequence. The upstream sequence encodes the same amino acid sequence as the sequence of the first cassette from its 5' end to the upstream codon and the downstream sequence encodes the same amino acid sequence as the sequence of the first amino acid cassette from the downstream codon to its 3' end. The first cassette is excised from the DNA molecule encoding at least the protein and replaced by the second cassette to produce a nucleic acid molecule encoding a hybrid protein, from which the hybrid protein is expressed.

The embodiments of the invention described above and diagrammed in Figures 11 to 15 provide methods for modifying a surface exposed loop of a protein to produce a hybrid protein including a target amino acid sequence

for eliciting an immune response, at a high efficiency.

In the embodiments where the hybrid protein is disposed on a hybrid picornavirus, the embodiments of the invention described above and diagrammed in Figures 11 to 15 provide methods for increasing the efficiency of obtaining a viable hybrid picornavirus with a hybrid protein comprising a modified surface exposed loop thereon.

Advantages of the hybrid picornaviruses of the present invention include:

- growth of the hybrid picornaviruses to a high titre;
- the capability to induce a strong and cross-reactive anti-chlamydial immune response at the same time as inducing a strong anti-polio immune response, either or both of which may include a mucosal immune response, when administered to mammals
- the hybrid picornaviruses may be administered as oral vaccines and may be administered in combination with one or more other immunogenic and/or immunostimulating molecules.
- the hybrid picornaviruses could not potentiate chlamydial disease by sensitizing vaccines because of the absence of the 57 kD SRP.
- the hybrid picornaviruses and other hybrid proteins may be obtained at high efficiency.

It is clearly apparent to one skilled in the art that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, treatment of chlamydial infections and the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

Vaccine Preparation and Use

In an embodiment, immunogenic compositions, suitable to be used as, for example, vaccines, may be prepared from hybrid picornavirus expressing at least one chlamydial epitope and capable at inducing antibodies in a subject immunized therewith immunoreactive with at least three different chlamydial serovars as disclosed herein. The hybrid picornavirus may be viable, or inactivated by, for example, chemical treatment, such as formaldehyde treatment, and an attenuated hybrid picornavirus, such as an attenuated poliovirus, may be employed. For human vaccination, an inactivated or attenuated form of the hybrid picornavirus generally is employed. The immunogenic composition elicits an immune response which produces anti-chlamydial antibodies, including anti-MOMP antibodies, and such antibodies may be neutralizing and/or protective antibodies.

In one embodiment, immunogenic compositions may be prepared as injectables, as liquid solutions or emulsions. The hybrid picornavirus may be mixed with pharmaceutically-acceptable excipients which are compatible therewith. Excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Methods of achieving adjuvant effect for the immunogenic composition include the use of agents, such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to 0.1 percent solution in phosphate buffered saline. Such immunogenic compositions may be administered parenterally, by injection subcutaneously or intramuscularly.

Alternatively, and in a preferred embodiment, the immunogenic compositions comprising hybrid picornaviruses

formed according to the present invention, may be delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols and triglycerides. Oral formulations may include normally employed incipients, such as pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions may take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 1 to 95% of the hybrid picornavirus.

The immunogenic compositions and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be immunized, including, for example, the capacity of the individual's immune system to synthesize antibodies. Precise amounts of immunogenic compositions required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the hybrid picornavirus. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the immunogenic composition may also depend on the route of administration and will vary according to the size of the subject.

The nucleic acid molecules encoding the hybrid picornavirus expressing at least one chlamydial epitope and capable of inducing antibodies in a subject immunized

therewith immunoreactive with at least three different chlamydial serovars may also be used directly for immunization by administration of the DNA directly, for example by injection for genetic immunization. Processes for the direct injection of DNA into test subjects for genetic immunization are described in, for example, Ulmer *et al* (ref. 36).

Immunoassays

The hybrid picornaviruses of the present invention are useful as antigens in immunoassays, including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of anti-chlamydia antibodies. In ELISA assays, the hybrid picornavirus is immobilized onto a selected surface. After washing to remove incompletely adsorbed hybrid picornavirus, a nonspecific protein, such as a solution of bovine serum albumin (BSA) or casein, that is known to be antigenically neutral with regard to the test sample, may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials to be tested, in a manner conducive to immune complex (antigen/antibody) formation. This procedure may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate, for example, from about 2 to 4 hours, at temperatures, for example, of the order of 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The

washing procedure may include washing with a solution, such as PBS/Tween, or a borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound hybrid picarnovirus, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins. To provide detecting means, the second antibody may have an associated activity, such as an enzymatic activity that will generate, for example, a colour development upon incubating with an appropriate chromogenic substrate. Quantification may then be achieved by measuring the degree of colour generation using, for example, a visible spectra spectrophotometer.

In an additional diagnostic embodiment, the hybrid picornavirus expressing at least one chlamydial epitope and capable of inducing antibodies in a subject immunized therewith immunoreactive with at least three different chlamydial serovars has utility in the generation of chlamydia-specific antibodies following immunization of a host with the hybrid picarnovirus. Such chlamydia-specific antibodies can be used to specifically determine the presence of chlamydial organisms in a test sample. Thus, the chlamydia-specific antibodies are contacted with a sample, such as clinical or biological materials to be tested, for example, cells grown in vitro, in a manner conducive to immune complex (antigen/antibody) formation. Following formation of specific immunocomplexes between the test sample containing chlamydial organisms and the chlamydia-specific antibodies produced by immunization of a host with the hybrid picornavirus produced according to the present invention, and subsequent washing, the occurrence, and

even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody, as described above.

BIOLOGICAL DEPOSITS

Certain biological materials are described and referred to herein that have been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland USA pursuant to the Budapest Treaty and prior to the filing of this application. The deposited plasmids will become available to the public upon grant of a patent based upon the United States patent applications. The invention described and claimed herein is not to be limited in scope by the plasmids deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent plasmids that can be used to produce equivalent hybrid picornaviruses as described in this application are within the scope of the invention.

DEPOSIT SUMMARY

Plasmid	Virus	ATCC Designation	Date Deposited
pT7Ct7	PV1-Ct7	75450	April 22, 1993
pt7Ct8	PV1-Ct8	75451	April 22, 1993
pT7CtIVA1	PV1-CtIVA	75735	April 13, 1994
pt7CtIVB4	PV1-CtIVB	75736	April 13, 1994
pt7CtIVC9	PV1-CtIVC	75737	April 13, 1994

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been

employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Example 1

This Example illustrates the construction of picornaviruses expressing neutralisation epitopes of C.trachomatis.

A cDNA clone of bases 1175 to 2956 of the poliovirus type 1, Mahoney strain (PV1-M) genome was cut with restriction enzymes Sal I and Hind III. These enzymes excise a fragment containing bases 2754 to 2786, which encodes PV1-M amino acids 1094 to 1102, as shown in Figures 1 and 6. In this application, we use the four-digit code for poliovirus amino-acids; for example, 1095 is amino acid 95 of capsid protein VP1. New hybrid cDNA clones encoding both poliovirus and chlamydia amino-acid sequences were constructed by replacing the excised fragment with synthetic oligonucleotides coding for amino acids from the MOMPs of C.trachomatis serovar A or D. The new hybrid cDNA clones were cut with restriction enzymes Nhe I and SnaB I, which excise a hybrid fragment, including the chlamydial DNA sequences, from poliovirus base 2471 to 2956. A cDNA clone, for example pT7XLD or pT7CMCB, of the entire genome of PV1-M was cut with Nhe I and SnaB I to excise a fragment from poliovirus base 2471 to 2956. This was then replaced with a hybrid fragment including the chlamydial DNA sequences to produce a hybrid cDNA clone of the genome of PV1-M with bases 2754 to 2786 replaced by bases encoding a hybrid BC loop including C.trachomatis amino acids, as shown in Figures 1 and 6.

The plasmid pT7XLD and clones derived from pT7XLD, such as pT7CMCB, contain a promoter sequence for the enzyme T7 RNA polymerase at the 5′ end of the PV1-M cDNA. RNA transcripts of the PV1-M cDNA, including any bases encoding C.trachomatis amino acids, were prepared using T7 RNA polymerase as described by van der Werf et al

(ref. 37). Transfection of Vero cells with these RNA transcripts produced six viable hybrid viruses, designated PV1-Ct5, PV1-Ct7, PV1-Ct8, PV1-CtIVA, PV1-CtIVB and PV1-CtIVC. Transcripts of cDNAs pT7Ct2 and pT7Ct4 were apparently non-infectious. Transfection with transcripts of pT7XLD yielded a transfection-derived wild-type poliovirus designated PV1-XLD. The identities of polioviruses PV1-Ct7, PV1-Ct8, PV1-CtIVA, PV1-CtIVB, PV1-CtIVC and PV1-XLD were confirmed by sequencing virion RNA through the region coding for the replaced amino-acids.

Single-step growth curves of hybrid polioviruses expressing chlamydial immunostimulatory epitopes were determined. Virus was grown and recovered as described previously (ref. 28) and then titrated by plaque assay. Plaque assays were performed as described by Emini E.A. et al (ref. 13). Vero cells were used as the substrate for all plaque assays.

PV1-Ct7 and PV1-Ct8 produced plaques respectively similar in size to or slightly smaller than PV1-XLD. In a single-step growth cycle PV1-Ct7, PV1-Ct8, PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC were slightly impaired in comparison to PV1-XLD (see Figures 2 and 7). PV1-XLD grew faster than PV1-Ct8, which grew faster than PV1-Ct7. Similarly, PV1-XLD grew faster than PV1-CtIVB and PV1-CtIVC, which grew faster than PV1-CtIVA.

The antigenic characteristics of PV1-Ct7 and PV1-Ct8 are shown in Table 1. Both were neutralised by monoclonal antibody (mAb) A-20, which is specific for the VAGLEK (SEQ. ID NO: 1) epitope from VDI of C.trachomatis serovar A MOMP, indicating that this epitope is expressed in a recognisable form on both hybrids. Significantly, the hybrids were also neutralised by convalescent sera from infected primates, indicating that the VAGLEK (SEQ. ID NO: 1) epitope was expressed in a form recognisable during a natural infection. The hybrids induced high-

titer antisera to poliovirus, and were neutralisable by anti-PV1 antisera, indicating that the changes in polio NAg I had not significantly affected other antigenic sites on the virus.

The antigenic characteristics of PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC are shown in Table 4 (the Tables appear at the end of the disclosure). All were neutralised by monoclonal antibody (mAb) DIII-A3, which is specific for the LNPTIAG (SEQ. ID NO: 9) epitope from VDIV of C.trachomatis serovars A, B, Ba, L1, L2, L3, D, E, F, and G MOMPs, indicating that this epitope is expressed in a recognisable form on these hybrids. Significantly, these hybrids were also neutralised by convalescent sera from infected primates, indicating that the LNPTIAG (SEQ. ID NO: 9) epitope was expressed in a form recognisable during a natural infection. The hybrids induced high-titer antisera to poliovirus, and were neutralisable by anti-PV1 antisera, indicating that the changes in polio NAg I had not significantly affected other antigenic sites on the virus.

Example 2

This Example illustrates the use of picornavirus hybrids to induce high titer antisera against C.trachomatis.

Rabbits were inoculated with CsCl-purified PV1-Ct7 (rabbits #13 to #16), PV1-Ct8 (rabbits #17 to #20) or PV1-XLD (rabbits #9 to 12), or with purified serovar A MOMP (rabbits #5 to #8), or with synthetic peptide A8-VDI (rabbits #1 to #4), or with density gradient purified formalin killed C.trachomatis serovar A elementary bodies (EBs) (rabbits #EB13 and #EB14). The synthetic peptide A8-VDI seq ID, a 42-mer, contains a MOMP T-helper cell epitope and the VAGLEK (SEQ. ID NO: 1) B-cell epitope from VDI of serovar A MOMP (ref. 33). Note that, although the viruses used were live, poliovirus does not replicate in rabbits and that any response observed is

effectively the response to an inactivated antigen. The dose of virus per inoculation was 2.5 x 10^8 pfu, which was determined from A_{260} values to be approximately 3.0 x 10^{11} virions. This is equivalent to 0.5 pmol of virus or 30 pmol of the VAGLEK (SEQ. ID NO: 1) epitope, since each virion expresses 60 copies of the epitope. Similarly, the dose of the VAGLEK (SEQ. ID NO: 1) epitope per inoculation of serovar A MOMP or A8VD1 was 30 pmol, approximately equal to 1.2ug of MOMP and 0.16ug of peptide. Both MOMP and peptide express one copy of the epitope. The dose of chlamydial EBs per inoculation was 1.3 x 10^9 inclusion forming units, which was determined from direct particle counting to be approximately 3.25 x 10^{11} EBs. There are approximately 10^5 MOMP molecules per EB, so this dose of EBs is equivalent to approximately 3.25 x 10^{16} molecules of MOMP, or about 54 nmoles of the VAGLEK (SEQ. ID NO: 1) epitope.

For all antigens except chlamydial EBs, the following inoculation schedule was used. On day 0, rabbits were inoculated with one dose of antigen in Freund's complete adjuvant subcutaneously on the back, and simultaneously with one dose in saline intramuscularly. On days 14 and 28, the rabbits were inoculated with one dose in Freund's incomplete adjuvant subcutaneously on the back. Blood was taken prior to the first inoculation and after 42 days.

For chlamydial EBs, the following inoculation schedule was used. On day 0, rabbits were inoculated with one dose of antigen in Freund's complete adjuvant injected subcutaneously on the nap of the neck. On day 45, rabbits were inoculated with one dose of antigen in Freund's incomplete adjuvant subcutaneously on the nap of the neck. Blood was taken prior to the first inoculation and after 49 days.

The sera from rabbits immunized with PV1-Ct7, PV1-Ct8 and PV1-XLD were tested by western blotting against

trachoma serovars A, B and C to determine if they recognised the MOMP and to define specificity of the anti-MOMP response. Purified serovar A, B and C EBs were solubilized in sample buffer and electrophoresed on a 12.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). SDS-PAGE-separated chlamydial polypeptides were transferred to nitrocellulose paper (NCP) and incubated with rabbit antisera diluted 1:200 in phosphate buffered saline (PBS) containing 1% dry milk and 0.5% tween-20. Following overnight incubation with the antisera, the NCP was washed thoroughly in PBS Tween-20, probed with ^{125}I-labelled protein A (Amersham,Inc.), washed again with PBS-Tween 20, then dried. Radiolabelled chlamydial proteins were visualised by autoradiography.

The results obtained are shown in Figure 3. The panels in Figure 3 show, from top left to right, a Coomassie Brilliant Blue (CBB)-stained gel showing MOMP from serovars A, B and C, then the reaction with the three MOMPs of anti-PV1-Ct7 antisera from rabbits 13, 14, 15 and 16 (1:200 dilution), and from bottom left to right, the reaction with the three MOMPS of pooled anti-PV1XLD antisera and of anti-PV1-Ct8 antisera from rabbits 17, 18, 19, and 20. Rabbit sera raised against PV1-Ct7 or PV1-Ct8 reacted very intensely with the homotypic serovar A MOMP, except for serum from rabbit #19, which reacted with serovar A MOMP relatively weakly. Cross reactivity against heterologous MOMPs was observed with some sera. Sera from rabbits #13, 18 and 20 reacted with serovar C MOMP. Sera raised against PV1-XLD did not react with the MOMPS of any of the three trachoma serovars.

The same sera were tested in a dot blot assay to determine if they could recognise MOMP in the context of intact chlamydial elementary bodies (EBs) (see Figure 4). Viable C.trachomatis EBs were adsorbed to nitrocellulose paper and incubated with the antisera diluted 1:200 in

phosphate-buffered saline. EBs of C.trachomatis C complex serovars A, C, H, I, and J, and Intermediate complex serovars K, B, and F were used in the assay. Antibodies bound to the surface of the EBs were detected by incubation with ^{125}I-labelled protein A. As shown in Figure 4, sera from rabbits #13, 14, 15 (PV1-Ct7), 17, 19 and 20 (PV1-Ct8) reacted with EBs from serovars A, C, I, and J, while the serum from rabbit #18 (PV1-Ct8) reacted with EBs of serovars A, C, I, J, and K, and the serum from rabbit #16 (PV1-Ct7) reacted with EBs of serovar A. Sera raised against PV1-XLD did not react with EBs of any serovar tested.

These results show that PV1-Ct7 and PV1-Ct8 are capable of inducing antibodies which react strongly with serovar A MOMP and intact EBs, and which are cross reactive with MOMP and EBs of several different chlamydial serovars.

The specificity and antibody titers of the anti-PV1-Ct7 sera were further tested by ELISA, and compared to rabbit antisera raised against purified serovar A MOMP, peptide A8-VDI or chlamydial EBs. The sera were tested against peptide A-VDI, which also contains the VAGLEK (SEQ. ID NO: 1) epitope from VD I of serovar A MOMP, and formalin-fixed serovar A EBs. The results of these assays are shown in Table 2. Rabbit antisera raised against the control virus, PV1-XLD, did not react with any of the chlamydial antigens at the highest concentration of sera tested (1:32). All four rabbits immunised with PV1-Ct7 produced high titered antibodies (8,192 - 16,384) against the peptide A-VDI and intact serovar A EBs. This showed that the antibodies raised against PV1-Ct7 recognised chlamydial epitopes in the context of MOMPs native configuration on the chlamydial surface. Anti-PV1-Ct7 sera also exhibited considerable cross reactivity against serovar C by ELISA. However, unlike the anti-A response, the anti-C response was

clearly more variable among immunised rabbits, with titers ranging from 256 to 8,192. None of the anti PV1-Ct7 sera reacted by ELISA with serovar B at a dilution of 1:32.

In contrast to these results, rabbits inoculated with a thousand-fold molar excess of chlamydial EBs responded with four-fold lower titers (4096) against the peptide A-VDI, and four-fold higher titers (32768 and 65536) against serovar A EBs. The latter titers are attributable in part to non-neutralising antibodies reactive with epitopes other than the VAGLEK (SEQ. ID NO: 1) epitope. Rabbits inoculated with a dosage of purified serovar A MOMP or A8-VDI that contained the same molar amount of the VAGLEK (SEQ. ID NO: 1) epitope as the dosage of PV1-Ct7 produced markedly lower and more variable antibody responses against both peptide A-VDI and serovar A EBs (Table 2). It has previously been found that significantly greater (100 to 200 fold) doses of purified MOMP and A8-VDI can induce antibody responses comparable in titer and specificity to those induced here by PV1-Ct7 (ref. 33). These findings very clearly demonstrate the superior immunogenicity of PV1-Ct7 in its ability to induce anti-chlamydial antibodies.

The rabbit anti-PV1-Ct7 sera were tested for their ability to neutralize chlamydial infectivity for HaK cells. Hamster kidney (HaK) cells (10^5 cells/well) were grown overnight in flat-bottomed 96-well microtiter plates (Linbro, Flow Laboratories Inc., McClean, VA). Serovar A and C EBs were diluted in 0.25M sucrose, 10mM sodium phosphate, 5mM L-glutamic acid, pH 7.2 (SPG). To determine serum neutralisation titers, two-fold dilutions of heat-inactivated (56°C for 30 min) sera were made in SPG. 100ul of diluted serum was mixed with 100ul of EBs in SPG at 6.6×10^5 inclusion forming units (IFU) per ml, then incubated at 37°C for 30min. A 50ul volume was then inoculated in triplicate onto HaK monolayers. The

monolayers were incubated at 37°C for 2 hr, washed once with 100ul Hanks balanced salt solution (HBSS) then fed with 200ul Minimal Essential Medium containing 10% fetal calf serum and 1ug/ml cycloheximide. Cells were incubated at 37°C for 48 to 70 hours, washed with HBSS, and fixed with absolute methanol. To visualize chlamydial inclusions, methanol-fixed cells were incubated with a murine mAb to chlamydial lipopolysaccharide then with a fluorescein-conjugated rabbit anti-mouse antibody. Fluorescent chlamydial inclusions were counted in 10 microscopic fields-of-view at 100X magnification from triplicate wells. In neutralisation assays the percent reduction of IFUs was calculated in comparison to pre-immune serum controls. All four rabbit anti-PV1-Ct7 sera exhibited consistent high-titered neutralising activity against the homotypic serovar A (Figure 5), in that the serum dilution resulting in a 50% reduction in serovar A infectivity (ND_{50}) was approximately 1:2,048 for each serum. These sera also showed neutralising activity against serovar C. However, with the exception of rabbit #13, which had an ND_{50} of 1:2,048, the anti-C neutralising titers were lower and more variable than the anti-A titers. These results show that chlamydial epitopes expressed on a hybrid poliovirus can induce antibodies which are functionally relevant in neutralising chlamydial infectivity.

To determine if the anti-PV1-Ct7 sera were capable of neutralising chlamydial infectivity for cells which are more typically colonised by chlamydiae in the context of a naturally occurring ocular infection, we tested the ability of rabbit anti-PV1-Ct7 sera to passively neutralise the infectivity of serovar A EBs for the conjunctivae of cynomolgus monkeys, an animal model that closely resembles human chlamydial ocular infection (refs. 44, 45). As shown in Table 3, anti-PV1-Ct7 sera were passively neutralising in this context. All three

monkeys inoculated with chlamydiae incubated with pre-immune sera shed chlamydiae from their conjunctivae at 3, 7, and 14 days post challenge. These monkeys also developed a mild follicular conjunctivitis characterized by conjunctival hyperemia and follicle formation. In contrast, those monkeys that received chlamydiae incubated with anti-PV1-Ct7 sera were culture negative through day 14 post-challenge, and none developed clinical signs of conjunctivitis. These results show that chlamydial epitopes expressed on a hybrid poliovirus can induce neutralising antibodies which are functionally relevant in an appropriate animal model.

Example 3

This Example further illustrates the use of picornavirus hybrids to induce high titer antisera cross-reactive against multiple C.trachomatis serovars.

Rabbits were inoculated with CsCl-purified PV1-XLD (rabbits #21 to #23), PV1-CtIVA (rabbits #24 to #26), PV1-CtIVB (rabbits #27 to #29) or with PV1-CtIVC (rabbits #30 to 32). Note that, although the viruses used were live, poliovirus does not replicate in rabbits and that any response observed is effectively the response to an inactivated antigen. The dose of virus per inoculation was 2.5×10^8 pfu, which was determined from A_{260} values to be approximately 3.0×10^{11} virions. This is equivalent to 0.5 pmol of virus or 30 pmol of the LNPTIAG (SEQ. ID NO: 9) epitope, since each virion expresses 60 copies of the epitope.

The following inoculation schedule was used. On day 0, rabbits were inoculated with one dose of antigen in Freund's complete adjuvant subcutaneously on the back, and simultaneously with one dose in saline intramuscularly. On days 14 and 28, the rabbits were inoculated with one dose in Freund's incomplete adjuvant subcutaneously on the back. Blood was taken prior to the first inoculation and after 42 days.

The sera from rabbits immunized with PV1-CtIVA, PV1-CtIVB, PV1-CtIVC and PV1-XLD were tested by western blotting (using the method described in Example 2) against all fifteen trachoma serovars to determine if they recognised the MOMPs and to define specificity of the anti-MOMP response (see Figure 8). Rabbit sera raised against PV1-CtIVA, PV1-CtIVB, PV1-CtIVC reacted strongly with all C.trachomatis MOMPs. Sera raised against PV1-XLD did not react with the any of the MOMPs.

The same sera were tested in a dot blot assay (using the method described in Example 2) to determine if they could recognise MOMP in the context of intact chlamydial elementary bodies (EBs) (see Figure 9). In a dot blot assay, sera from rabbits #24 to 32 consistently reacted with EBs from serovars L1, L2, L3, D, E and K. Several sera reacted less intensely with EBs from serovars A, B, Ba, F and G. Sera raised against PV1-XLD did not react with EBs of any serovar (not shown).

These results show that PV1-CtIVA, PV1-CtIVB, PV1-CtIVC are capable of inducing antibodies which react strongly with serovar D MOMP and intact EBs, and which are cross reactive with MOMPs and EBs of several different chlamydial serovars.

The rabbit anti-PV1-CtIVA, -PV1-CtIVB, and -PV1-CtIVC sera were pooled according to immunising antigen and tested for their ability to neutralise chlamydial infectivity for HaK cells (as described in Example 2). The results are shown in Figure 10. All three pools exhibited high-titered neutralising activity against C.trachomatis serovars Ba, D, E, G, and K. Anti-PV1-CtIVC also neutralised serovar A. Pooled anti-PV1-XLD did not exhibit any neutralising activity. These results show that chlamydial epitopes expressed on a hybrid poliovirus can induce antibodies which are functionally relevant in neutralising infectivity of several different chlamydial serovars.

Example 4

Particular embodiments of the present invention provide methods of modifying a virus protein having a surface exposed loop to produce a hybrid protein. This Example illustrates the construction of a hybrid poliovirus capsid protein VP1 incorporating an epitope from C. trachomatis, employing such methods.

Crystallographic data for poliovirus capsid protein VP1 was obtained from the Brookhaven Protein Data Bank (Protein Data Bank entry 2PLV). Temperature factors for each amino acid residue in the BC loop of VP1 were analyzed, and the region of the loop containing those residues with the highest temperature factors was identified as the region of the loop of VP1 having the greatest mobility. This region was considered appropriate for insertion of a target amino acid sequence for eliciting an immune response. In the present Example, the region including amino acids 1097 to 1102, (all of which had temperature factors above 50) was deemed to have mobility adequate for accommodation of additional amino acids of the target without disrupting the overall folding and stability of VP1.

The target chlamydial sequence was TTSDVAGLEKDPVA (SEQ. ID NO: 26). This selected amino acid was examined and the amino terminal amino acid, T, was identified as being the same as amino acid 1098, also T, in the region in the BC loop of VP1 having the greatest mobility. Accordingly, it was determined to delete amino acids 1098 to 1102 (TTNKD; SEQ. ID NO: 25) from the region of the BC loop containing those residues with a highest temperature factors and to insert the target chlamydial amino acid sequence.

This particular insertion was effected using a nucleic acid cassette encoding the target amino acid sequence and a nucleic acid cassette encoding part of capsid protein VP1 as shown in Figures 1 and 15. In this

Example, the upstream codon is the codon encoding amino acid 1097, S, in the BC loop of VP1, and the downstream codon is the codon encoding amino acid 1103, K, in the BC loop of VP1.

Using cassette mutagenesis, restriction endonucleases and synthetic oligonucleotides, the sequence encoding amino acids 1098 to 1102 (TTNKD; SEQ. ID NO: 25) was deleted from the nucleic acid molecule encoding capsid protein VP1 and replaced with a sequence encoding the target amino acid sequence.

The DNA molecule encoding the capsid protein VP1 was the plasmid pT7XLD, and the hybrid poliovirus capsid protein VP1 was expressed as part of poliovirus PV1-Ct7, as described in Example 1, to produce a viable hybrid picornavirus having a hybrid protein with a hybrid protein comprising a modified surface exposed loop thereon.

SUMMARY OF INVENTION

In summary of this disclosure, the present invention provides novel hybrid picornaviruses which express chlamydial epitopes, useful in vaccines against both poliomyelitis and chlamydial diseases, and in the preparation of immunological reagents and in the specific identification of chlamydia. The disclosure also provides methods for modifying proteins having surface exposed loops of known sequences to produce hybrid proteins. Modifications are possible within the scope of the invention.

TABULAR DATA

Table 1

Table 1 shows neutralisation of C.trachomatis and of polio-chlamydia hybrids by anti-chlamydial and anti-hybrid sera and monoclonal antibodies.

MAb A-20 is specific to C.trachomatis serovar A. The hybridoma secreting mAb A-20 was generated from mouse splenocytes following intraperitoneal immunization with intact formalin-killed serovar A EBs. MAb A-20 binds to intact serovar A EBs by dot-immunoblot, and also neutralises their infectivity. It is MOMP specific, and its binding site maps to the hexameric VAGLEK (SEQ. ID NO: 1) sequence located within VDI of serovar A MOMP (refs. 3, 42). MAb B-B6 is a serovar B MOMP-specific antibody and was used as a negative control in virus neutralisation assays; it was prepared in the same way as A-20, and is of the same isotype (ref. 43). For neutralisation assays, both were used at a starting concentration of 1mg/ml.

Monkey convalescent sera were obtained from cynomolgus monkeys that had recovered from a primary conjunctival infection with serovar A. Pre-immune sera were taken prior to experimental infection and convalescent sera were taken 45 days post-infection, approximately two weeks after the monkeys became culture negative and were free of any clinical signs (conjunctival hyperemia and follicles) of disease. The sera were heat inactivated at 56°C for 30 minutes prior to assay.

Rabbit sera were obtained as described in the second example. Sera were titrated for their ability to neutralise 100 50% tissue culture infectious doses of virus. Vero cells were used as the substrate for all virus neutralisation assays. Additionally, sera were titrated for their ability to neutralise infectious chlamydiae as described in Example 2.

Table 1 Neutralisation of C.trachomatis and of polio/chlamydia hybrids by anti-chlamydia and anti-hybrid sera and monoclonal antibodies.

SERUM[a]/ANTIBODY	NEUTRALISING TITER[b] AGAINST			
	PV1-XLD	PV1-Ct7	PV1-Ct8	C.trachomatis Serovar A
Monoclonals				
B-B6	<4	<4	<4	<16
A-20	<4	>2048	601	2048
Monkey convalescent				
831	<2	6	10	128
840	<2	23	16	128
939	<2	23	10	64
Rabbit[c]				
anti-PV1-XLD	20,000	14,125	12,589	F4[d]
anti-PV1-Ct7	28,183	ND[e]	ND	F4
anti-PV1-Ct8	56,234	ND	ND	F4

a. All monkey prebleeds had titers of <2 and all rabbit prebleeds had titers of <4 against PV1-XLD, PV1-Ct7 and PV1-Ct8. Monkey prebleeds had titres of <16 against C.trachomatis.

b. Anti-virus titers are the reciprocal dilution of serum/mAb giving a 50% endpoint in a neutralisation assay versus 100 $TCID_{50}$ of virus. Anti-chlamydial titers are the reciprocal of the highest dilution of serum/mAb giving at least 50% reduction in IFU assayed on HaK cells. Dilutions of monoclonal antibodies refer to a starting concentration of 1 mg/ml.

c. Pooled sera from final (42 days) bleed.

d. F4 = Pooled sera not tested, see figure 4 for data on individual sera.

e. ND = not done

Table 2

Table 2 shows the immunogenicity of polio-chlamydia hybrid PV1-Ct7 as determined by ELISA assay.

Immunlon 2 microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated overnight at 4°C with 100ul of peptide A-VDI (MOMP residues 66-85) or formalin-killed C.trachomatis serovar A EBs at 10ug/ml in 0.05M Tris buffer, pH 7.5 containing 0.15M NaCl. Serial two-fold dilutions of rabbit sera were tested in duplicate. Rabbit IgG was detected using an anti-rabbit IgG-alkaline phosphatase conjugate (heavy and light chain specific; Zymed Laboratories Inc., San Francisco, CA) followed by substrate (5mg p-nitrophenyl phosphate in 10ml of 0.1M 2:2 amino-2-methyl-1:3-propandiol, pH 10.3). Absorbence at 405nm was measured with an ELISA reader (Bio-Rad Laboratories, Richmond, CA).

Table 2. Immunogenicity of polio-chlamydia hybrid PV1-Ct7 as determined by ELISA assay.

Rabbit antisera raised against	Rabbit #	ELISA titer against[a] Peptide A-VDI	Serovar A EBs
PV1-Ct7	13	16384	8192
	14	8192	8192
	15	16384	8192
	16	16384	16384
PV1XLD	9	<32	<32
	10	<32	<32
	11	<32	<32
	12	<32	<32
Serovar A MOMP	5	256	<32
	6	512	<32
	7	1024	128
	8	128	32
Peptide A8-VDI	1	<32	<32
	2	256	32
	3	1024	<32
	4	128	<32
Serovar A EBs	EB13	32768	4096
	EB14	65536	4096

a = ELISA titers are expressed as the reciprocal dilution giving an absorbance reading of 0.3. Absorbance values for preimmune sera at a dilution of 1:32 were 0.1 or less.

Table 3

Table 3 shows that the rabbit anti-PV1-Ct7 sera is capable of neutralising chlamydial infectivity for cells which are more typically colonised by chlamydiae in the context of a naturally occurring ocular infection. Heat-inactivated (56°C, 30 min) pools of preimmune and immune sera from the PV1-Ct7-immunised rabbits (#s 13, 14, 15, and 16) were prepared and tested for their ability to neutralise C.trachomatis serovar A infectivity for the monkey conjunctivae. 300ul of 0.25M sucrose, 10mM sodium phosphate, 5mM L-glutamic acid, pH7.2 (SPG) containing 10^6 IFU of serovar A EBs was mixed with 300ul of a 1:100 dilution in SPG of pooled immune or preimmune serum and incubated at 37°C for 30 minutes. Immediately thereafter 20ul aliquots of the serum/chlamydia mixtures were inoculated onto the upper and lower conjunctivae of both eyes of three cynomolgus monkeys. Clinical disease and the presence of viable chlamydiae were determined on days 7, 14, and 21 post-challenge.

Table 3. Passive neutralisation of chlamydial infectivity for monkey conjunctivae by anti-PV1-CT7 sera.

Sera and monkey #	Chlamydial culture[a] on day post-challenge			Clinical disease score[b] of challenged monkeys on day post-challenge		
	7	14	21	7	14	21
Preimmune 842	+++	+	++	+/-	++/-	++/++
Preimmune 880	+	+++	++	+/-	++/++	++/+++
Preimmune 686	+++	++	+++	+/-	++/-	++/++
Immune 918	-	-	-	-/-	-/-	-/-
Immune 939	-	-	-	-/-	-/-	-/-
Immune 969	-	-	-	-/-	-/-	-/-

a. Culture results are expressed as the average number of chlamydial IFU per 3 x 10^5 HeLa 229 cells isolated from the conjunctivae of both eyes; - = 0 IFU, + = 1 to 10 IFU, ++ = 10 to 100 IFU, +++ = >100 IFU.

b. The clinical disease results represent aggregate scores from both eyes of each monkey. Clinical disease scores for hyperemia are shown before the slash, those for follicle formation after the slash. The scores were determined as follows. For hyperemia: - = none; + = mild; ++ = moderate; +++ = severe. For follicle formation: - = none; + = just present (1 to 3); ++ = obviously present (4 to 10); +++ = grossly present (>10).

Table 4

Table 4 shows neutralisation of polio-chlamydia hybrids by anti-chlamydial sera and monoclonal antibodies.

MAb A-20 is specific to C.trachomatis serovar A. The hybridoma secreting mAb A-20 was generated from mouse splenocytes following intraperitoneal immunization with intact formalin-killed serovar A EBs. MAb A-20 binds to intact serovar A EBs by dot-immunoblot, and also neutralises their infectivity. It is MOMP specific, and its binding site maps to the hexameric VAGLEK (SEQ. ID NO: 1) sequence located within VDI of serovar A MOMP (refs. 3, 42, 43). MAb DIII-A3 was prepared similarly, and its binding site includes the LNPTIAG (SEQ. ID NO: 9) sequence located within VDIV of serovar D MOMP (refs. 3, 42, 43). For neutralisation assays, both were used at a starting concentration of 1mg/ml.

Monkey convalescent sera were obtained from cynomolgus monkeys that had recovered from a primary cervical infection with serovar D. Pre-immune sera were taken prior to experimental infection and convalescent sera were taken following recovery. The sera were heat inactivated at 56°C for 30 minutes prior to assay.

Rabbit sera were obtained as described in the second example. Sera were titrated for their ability to neutralise 100 50% tissue culture infectious doses of virus, using the method of Golding et al (1976). Vero cells were used as the substrate for all virus neutralisation assays.

Table 4 Neutralization of polio/chlamydia hybrids expressing the chlamydial VDIV epitope "LNPTIAG" by anti-chlamydial sera and monoclonal antibodies.

VIRUS	SERA/McAb[a]					
(PV1-)	A-20	DIIIA-3	705(PB)	705(Con)	880(Con)	anti-PV1
XLD	<20[b]	<20	<10	<10	<10	28,973
CtIVA	<20	10,240	<10	299	75	17,782
CtIVB	<20	135	<10	423	80	40,960
CtIVC	<20	<20	<10	92	14	13,388

a. A-20 and DIIIA-3 are monoclonal antibodies specific for the VDI epitope VAGLEK (SEQ. ID No. 1) and the VDIV epitope LNPTIAG (SEQ. ID No. 9) respectively. Sera 705 and 880 are from monkeys infected cervically with C.trachomatis serovar D. Anti-PV1 is pooled rabbit anti-poliovirus type 1.

b. Titres are reciprocal dilutions giving 50% endpoints in a neutralisation assay versus 100 $TCID_{50}$ of virus.

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Table 5

Table 5 shows some examples of predicted functional variants of hybrid BC loops including chlamydial epitopes. A functional variant is defined as one which permits the construction of a viable hybrid picornavirus expressing at least one chlamydial epitope and capable of inducing, in a subject immunised therewith, antibodies immuno-reactive with at least three different chlamydial serovars.

The predicted functional variants contained in Table 5 can conveniently be tested by the procedure of:-

- constructing a hybrid cDNA clone of the genome of PV1-M with bases 2754 to 2786 replaced by bases encoding a predicted functional variant of a hybrid BC loop, as described in Example 1;
- preparing RNA transcripts of the genome of PV1-M with bases 2754 to 2786 replaced by bases encoding a predicted functional variant of a hybrid BC loop, as described in Example 1;
- transfecting said transcripts into Vero cells and observing for the production of viable hybrid viruses;
- immunising rabbits with said viable hybrid viruses and taking serum samples as described in Example 2; and
- testing said serum samples for antibodies immuno-reactive with at least three different chlamydial serovars in a dot blot assay as described in Example 2.

Any variant which does not permit the construction of a viable hybrid picornavirus expressing at least one chlamydial epitope and capable of inducing, in a subject immunised therewith, antibodies immuno-reactive with at least three different chlamydial serovars according to this test procedure is not a functional variant for the purposes of this application.

It will be readily apparent to one skilled in the art that other possible functional variants exist, for example,

variants in which conservative amino-acid changes are made in the sequence of a hybrid BC loop. All such possible variants may conveniently be tested for functionality as described here.

Table 5. Some examples of possible functional variants of hybrid BC loops, showing possible amino acid substitutions occurring in said variants. Variants of hybrid BC loops may conveniently contain at least one amino acid substitution. These examples are given for illustrative purposes only, and this table is not intended to be an exhaustive list of possible variants.

A) Some possible functional variants of a hybrid BC loop including a chlamydial epitope.

Original construct	ASTT [X] KDKL (SEQ. ID NOS: 33 and 34)
Possible substitutions of each amino acid[1]	****# #** TFIK NGR SPAE ES QINQ AQ N N PL L I VP S TR H K

B) Some possible functional variants of a hybrid BC loop including a chlamydial serovar A-specific epitope.

Original construct	[Z1] TTLNPTIAGAGDVK [Z2] (SEQ. ID NO: 11)
Possible substitutions of each amino acid	#*** *******# T CEE A K S V T A

C) Some possible functional variants of a hybrid BC loop including a chlamydial species specific-epitope.

Original construct	[Z1] TTSDVAGLEKDPVA [Z2] (SEQ. ID NO: 26)
Possible substitutions of each amino acid	#****** ******# K AED QN TT N T S K

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[1] Where [X] is a chlamydial immunostimulating epitope or a functional variant thereof, [Z1] and [Z2] are native BC loop sequences or functional variants thereof, * indicates a deletion, and # indicates a possible insertion of one or more amino acids on either side of the chlamydial immunostimulating epitope or functional variant thereof.

Possible substitutions of each amino acid in the original construct are shown in the column below each amino acid. For example, possible substitutions of S in the original

construct shown in A) include * (a deletion), F, P, I, N, or L. Variants of hybrid BC loops may conveniently contain substitutions of at least one amino acid.

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SEQUENCE LISTING

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(i) APPLICANT:
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(F) POSTAL CODE (ZIP): L0G 1T0

(ii) TITLE OF INVENTION: HYBRID PICORNAVIRUSES EXPRESSING CHLAMYDIAL EPITOPES

(iii) NUMBER OF SEQUENCES: 34

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Ala Gly Leu Glu Lys
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn Pro Ala Ser Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Gly Asp
1 5 10 15

Val Lys Asp

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asn Pro Ala Ser Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Gly Asp
1 5 10 15

Asp

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Pro Ala Ser Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Pro Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro Val Ala
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asn Pro Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro Lys Asp
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Pro Ala Ser Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro
1 5 10 15

Val Ala

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asn Pro Ala Ser Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro
1 5 10 15

Lys Asp

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR D

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Asn Pro Thr Ile Ala Gly
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR D

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Gly Asp Val Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR D

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Gly Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR D

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala Ser Thr Thr Asn Lys Asp Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala Ser Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro Val Ala
1 5 10 15

Lys Leu

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Ser Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro Lys Asp
1 5 10 15

Lys Leu

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ala Ser Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Gly Asp Val Lys
1 5 10 15

Asp Lys Leu

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ala Ser Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Gly Asp Asp Lys
1 5 10 15

Leu

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ala Ser Thr Thr Leu Asn Pro Thr Ile Ala Gly Ala Asp Lys Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Asn Pro Ala Ser Thr Thr Asn Lys Asp
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Val Ala Gly Leu Glu Lys Asp Pro Val Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Pro Ala Ser Thr Thr Asn Lys Asp
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Pro Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro Val Ala
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Pro Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Thr Thr Asn Lys Asp
1 5

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Thr Thr Ser Asp Val Ala Gly Leu Glu Lys Asp Pro Val Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Thr Thr Asn
1

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CHLAMYDIA TRACHOMATIS
(B) STRAIN: SEROVAR A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Asp Val Ala Gly Leu Glu Lys Asp
1 5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Thr Thr Asn Lys
1

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Pro Ala Ser Thr Thr Asn
1 5

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Val Asp Asn Pro Ala Ser Thr Thr Asn Lys Asp Lys Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: POLIOVIRUS
(B) STRAIN: TYPE 1 MAHONEY

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GTCGACAACC CAGCTTCCAC CACCAATAAG GACAAGCTT 39

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Ala Ser Thr Thr
1

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Lys Asp Lys Leu
1

CLAIMS

What we claim is:

1. A hybrid picornavirus expressing at least one chlamydial epitope and capable of inducing antibodies immuno-reactive with at least three different Chlamydia serovars.

2. The hybrid picornavirus of claim 1, wherein the at least three different Chlamydia serovars are at least three different Chlamydia trachomatis serovars.

3. The hybrid picornavirus of claim 2 wherein the hybrid picornavirus is a hybrid poliovirus.

4. The hybrid picornavirus of claim 3, wherein the at least one chlamydial epitope comprises a linear amino acid sequence of between about 5 and about 15 amino acids.

5. The hybrid picornavirus of claim 4, wherein the at least one chlamydial epitope comprises an epitope of the major outer membrane protein of Chlamydia trachomatis.

6. The hybrid picornavirus of claim 5, wherein the at least one epitope of the major outer membrane protein comprises a serovar-specific epitope.

7. The hybrid picornavirus of claim 6 wherein the epitope of the major outer membrane protein comprises a variable domain I sequence.

8. The hybrid picornavirus of claim 7, wherein the variable domain I sequence includes an amino acid sequence selected from VAGLEK (SEQ. ID NO: 1), PTTSDVAGLEKDP (SEQ. ID NO: 24), TTSDVAGLEKDPVA (SEQ. ID NO: 26), TTSDVAGLEKDP (SEQ. ID NO: 10) or a functional variant thereof.

9. The hybrid picornavirus of claim 5, wherein the at least one epitope of the major outer membrane protein comprises a species-specific epitope.

10. The hybrid picornavirus of claim 9 wherein the species-specific epitope comprises a variable domain IV sequence.

11. The hybrid picornavirus of claim 10, wherein the variable domain IV sequence includes an amino acid sequence selected from LNPTIAG (SEQ. ID NO: 9), TTLNPTIAGAGDVK (SEQ. ID NO: 11), TTLNPTIAGAGD (SEQ. ID NO: 12), TTLNPTIAGA (SEQ. ID NO: 13) or a functional variant thereof.

12. The hybrid picornavirus of claim 5, wherein the at least one chlamydial epitope is contained within a hybrid BC loop sequence of poliovirus capsid protein VP1.

13. The hybrid picornavirus of claim 12, wherein the hybrid BC loop comprises a BC loop sequence of poliovirus capsid protein VP1 extended by the chlamydial epitope.

14. The hybrid picornavirus of claim 13, wherein the at least one epitope of the major outer membrane protein comprises a serovar-specific epitope.

15. The hybrid picornavirus of claim 14, wherein the serovar-specific epitope comprises a variable domain I sequence.

16. The hybrid picornavirus of claim 15, wherein the variable domain I sequence includes an amino acid sequence selected from VAGLEK (SEQ. ID NO: 1), PTTSDVAGLEKDP (SEQ. ID NO: 24), TTSDVAGLEKDPVA (SEQ. ID NO: 26), TTSDVAGLEKDP (SEQ. ID NO: 10) or functional variants thereof.

17. The hybrid picornavirus of claim 16, wherein the hybrid BC loop includes an amino acid sequence selected from ASTTSDVAGLEKDPVAKL (SEQ. ID NO: 15), ASTTSDVAGLEKDPKDKL (SEQ. ID NO: 16) or a functional variant thereof.

18. The hybrid picornavirus of claim 17, wherein the functional variant is selected from those of Table 5.

19. The hybrid picornavirus of claim 17, wherein the hybrid loop includes the amino acid sequence ASTTSDVAGLEKDPVAKL (SEQ. ID NO: 15) and the hybrid poliovirus is PV1-Ct7.

20. The hybrid picornavirus of claim 17 wherein the hybrid loop includes the amino acid sequence ASTTSDVAGLEKDPKDKL (SEQ. ID NO: 16) and the hybrid poliovirus is PV1-Ct8.

21. The hybrid picornavirus of claim 12, wherein the at least one epitope of the major outer membrane protein comprises a species-specific epitope.

22. The hybrid picornavirus of claim 21, wherein the species-specific epitope comprises a variable domain IV sequence.

23. The hybrid picornavirus of claim 22, wherein the variable domain IV sequence includes an amino acid sequence selected from LNPTIAG (SEQ. ID NO: 9), TTLNPTIAGAGDVK (SEQ. ID NO: 11), TTLNPTIAGAGD (SEQ. ID NO: 12), TTLNPTIAGA (SEQ. ID NO: 15) or a functional variant thereof.

24. The hybrid picornavirus of claim 23, wherein the hybrid BC loop includes an amino acid sequence selected from ASTTLNPTIAGAGDVKDKL (SEQ. ID NO: 17), ASTTLNPTIAGAGDDKL (SEQ. ID NO: 18), ASTTLNPTIAGADKL (SEQ. ID NO: 19) or a functional variant thereof.

25. The hybrid picornavirus of claim 24, wherein the functional variant is selected from those of Table 5.

26. The hybrid picornavirus of claim 23, wherein the hybrid loop includes the amino acid sequence ASTTLNPTIAGAGDVKDKL (SEQ. ID NO: 17) and the hybrid poliovirus is PV1-CtIVA.

27. The hybrid picornavirus of claim 23, wherein the hybrid loop includes the amino acid sequence ASTTLNPTIAGAGDDKL (SEQ. ID NO: 18) and the hybrid poliovirus is PV1-CtIVB.

28. The hybrid picornavirus of claim 23, wherein the hybrid loop includes the amino acid sequence ASTTLNPTIAGADKL (SEQ. ID NO: 19) and the hybrid poliovirus is PV1-CtIVC.

29. An isolated nucleic acid molecule comprising at least a portion encoding a hybrid picornavirus expressing at least one chlamydial epitope and capable of inducing antibodies immuno-reactive with at least three different Chlamydia serovars.

30. The isolated nucleic acid molecule of claim 29, wherein the at least three different Chlamydia serovars are at least three different Chlamydia trachomatis serovars.

31. The isolated nucleic acid molecule of claim 30, wherein the hybrid picornavirus is a hybrid poliovirus.

32. The isolated nucleic acid molecule of claim 31, wherein the at least one chlamydial epitope comprises a linear amino acid sequence of between about 5 and about 15 amino acids.

33. The isolated nucleic acid molecule of claim 32, wherein the at least one chlamydial epitope comprises an epitope of the major outer membrane protein of Chlamydia trachomatis.

34. The isolated nucleic acid molecule of claim 32, wherein the at least one epitope of the major outer membrane protein comprises a serovar-specific epitope.

35. The isolated nucleic acid molecule of claim 34, wherein the epitope of the major outer membrane protein comprises a variable domain I sequence.

36. The isolated nucleic acid molecule of claim 35, wherein the variable domain I sequence includes an amino acid sequence selected from VAGLEK (SEQ. ID NO: 1), PTTSDVAGLEKDP (SEQ. ID NO: 24), TTSDVAGLEKDPVA (SEQ. ID NO: 26), TTSDVAGLEKDP (SEQ. ID NO: 10) or functional variants thereof.

37. The isolated nucleic acid molecule of claim 36, wherein the at least one epitope of the major outer membrane protein comprises a species-specific epitope.

38. The isolated nucleic acid molecule of claim 37, wherein the species-specific epitope comprises a variable domain IV sequence.

39. The isolated nucleic acid molecule of claim 38, wherein the variable domain I sequence includes an amino acid sequence selected from LNPTIAG (SEQ. ID NO: 9), TTLNPTIAGAGDVK (SEQ. ID NO: 11), TTLNPTIAGAGD (SEQ. ID NO: 12), TTLNPTIAGA (SEQ. ID NO: 13) or functional variants thereof.

40. The isolated nucleic acid molecule of claim 33, wherein the at least one chlamydial epitope is contained within a hybrid BC loop sequence of poliovirus capsid protein VP1.

41. The isolated nucleic acid molecule of claim 40, wherein the hybrid BC loop comprises a BC loop sequence of poliovirus capsid protein VP1 extended by the chlamydial epitope.

42. The isolated nucleic acid molecule of claim 40, wherein the at least one epitope of the major outer membrane protein comprises a serovar-specific epitope.

43. The isolated nucleic acid molecule of claim 42, wherein the epitope of the major outer membrane protein comprises a variable domain I sequence.

44. The isolated nucleic acid molecule of claim 43, wherein the variable domain I sequence includes an amino acid sequence selected from VAGLEK (SEQ. ID NO: 1), PTTSDVAGLEKDP (SEQ. ID NO: 24), TTSDVAGLEKDPVA (SEQ. ID NO: 26), TTSDVAGLEKDP (SEQ. ID NO: 10) or functional variant thereof.

45. The isolated nucleic acid molecule of claim 44, wherein the hybrid BC loop includes an amino acid sequence selected from ASTTSDVAGLEKDPVAKL (SEQ. ID NO: 15), ASTTSDVAGLEKDPKDL (SEQ. ID NO: 16) or a functional variant thereof.

46. The isolated nucleic acid molecule of claim 45, wherein the functional variant is selected from those of Table 5.

47. The isolated nucleic acid molecule of claim 45, wherein the hybrid loop includes the amino acid sequence ASTTSDVAGLEKDPVAKL (SEQ. ID NO: 15) and the DNA molecule is pT7Ct7.

48. The isolated nucleic acid molecule of claim 45, wherein the hybrid loop includes the amino acid sequence ASTTSDVAGLEKDPKDKL (SEQ. ID NO: 16) and the DNA molecule is pT7Ct8.

49. The isolated nucleic acid molecule of claim 29, wherein the at least one epitope of the major outer membrane protein comprises a species-specific epitope.

50. The isolated nucleic acid molecule of claim 49, wherein the species-specific epitope comprises a variable domain IV sequence.

51. The isolated nucleic acid molecule of claim 50, wherein the variable domain IV sequence includes an amino acid sequence selected from LNPTIAG (SEQ. ID NO: 9), TTLNPTIAGAGDVK (SEQ. ID NO: 11), TTLNPTIAGAGD (SEQ. ID NO: 12), TTLNPTIAGA (SEQ. ID NÓ: 13) or a functional variant thereof.

52. The isolated nucleic acid molecule of claim 50, wherein the hybrid loop includes the amino acid sequence ASTTLNPTIAGAGDVKDKL (SEQ. ID NO: 27) and the DNA molecule is pT7CtIVA1.

53. The isolated nucleic acid molecule of claim 50, wherein the hybrid loop includes the amino acid sequence ASTTLNPTIAGAGDDKL (SEQ. ID NO: 18) and the DNA molecule is pT7CtIVB4.

54. The isolated nucleic acid molecule of claim 50, wherein the hybrid loop includes the amino acid sequence ASTTLNPTIAGADKL (SEQ. ID NO: 19) and the DNA molecule is pT7CtIVC9.

55. An immunogenic composition comprising an immunoeffective amount of the hybrid picornavirus of any one of claims 1 to 28 or an isolated nucleic acid molecule of any one of claims 29 to 54 and a pharmaceutically acceptable carrier therefor.

56. The immunogenic composition of claim 55 formulated for mucosal or parenteral administration.

57. The immunogenic composition of claim 56 further comprising at least one other immunogenic or immunostimulating material.

58. The composition of claim 57, wherein the at least one other material is an adjuvant.

59. The composition of claim 58, wherein the adjuvant is aluminum phosphate or aluminum hydroxide.

60. The composition of claim 55 formulated as a vaccine for human use, wherein the hybrid picornavirus is attenuated or inactivated or the nucleic acid molecule encodes an attenuated picornavirus.

61. A method of immunizing a host comprising of administering thereto an immunogenic composition comprising an immunoeffective amount of the hybrid picornavirus of any one of claims 1 to 28 or any isolated nucleic acid molecule of any one of claims 29 to 54 and a pharmaceutically acceptable carrier therefor.

62. The method of claim 61, wherein the immunogenic composition is formulated for mucosal or parenteral administration.

63. The method of claim 62, wherein the immunogenic composition further comprises of at least one other immunogenic or immunostimulating material.

64. The method of claim 63, wherein at least one other material is an adjuvant.

65. The method of claim 64, wherein the adjuvant is aluminum phosphate or aluminum hydroxide.

66. The method of claim 61 wherein said host is a human and wherein the hybrid picornavirus is attenuated or

inactivated or the nucleic acid molecule encodes an attenuated picornavirus.

67. A method of determining the presence of chlamydia in a sample, comprising the steps of:

(a) immunizing a subject with the immunogenic composition of claim 55 to produce chlamydia-specific antibodies;

(b) contacting the sample with the chlamydia specific antibodies to produce complexes comprising chlamydia and chlamydia-specific antibodies; and

(c) determining production of the complexes.

68. An antibody immunoreactive with at least three different Chlamydia serovars producible by immunizing a host with the immunogenic composition of claim 55.

69. A diagnostic kit for detecting the presence of chlamydia in a sample, comprising:

(a) an antibody of claim 68;

(b) means for contacting the antibody with the sample to produce a complex comprising chlamydia and chlamydia specific antibody; and

(c) means for determining production of the complex.

70. A method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises:

(a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility;

(b) providing a first nucleic acid molecule encoding at least the protein;

(c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response;

(d) identifying a first amino acid proximate the amino terminus of the target sequence which is the same

as or functionally equivalent to a second amino acid in said identified portion;

(e) excising between one and all codons encoding said identified portion from the first nucleic acid molecule to produce an excised sequence the 5' codon of which encodes the second amino acid, an upstream codon which is the codon immediately preceding the excised sequence, and a downstream codon which is the codon immediately following the excised sequence;

(f) deleting zero or at least one codon from the 5' end of the second nucleic acid molecule to produce a third nucleic acid molecule the 5' codon of which is the codon encoding the first amino acid;

(g) inserting the third nucleic acid molecule between the upstream and downstream codons such that the 5' codon of the inserted third nucleic acid molecule encodes the first amino acid to produce a fourth nucleic acid molecule encoding the hybrid protein; and

(h) producing the hybrid protein from the fourth nucleic acid molecule.

71. A method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises:

(a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility;

(b) providing a first nucleic acid molecule encoding at least the protein;

(c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response;

(d) identifying a first amino acid proximate the amino terminus of the target sequence which is the same as or functionally equivalent to a second amino acid in said identified portion;

(e) deleting the codon encoding the first amino acid and all preceding codons from the 5' end of the second nucleic acid molecule to produce a third nucleic acid molecule;

(f) identifying in the first nucleic acid molecule an upstream codon which is the codon encoding the second amino acid and a downstream codon which is the immediately adjacent codon at the 3' end of the upstream codon;

(g) forming an insertion site in the first nucleic acid molecule between the upstream and the downstream codon;

(h) inserting the third nucleic acid molecule into the first nucleic acid molecule at the insertion site such that the 5' codon of the inserted third nucleic acid molecule encodes the first amino acid, to produce a fourth nucleic acid molecule encoding the hybrid protein; and

(i) producing the hybrid protein from the fourth nucleic acid molecule.

72. A method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises:

(a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility;

(b) providing a first nucleic acid molecule encoding at least the protein;

(c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response;

(d) identifying a first amino acid proximate the carboxy terminus of the target sequence which is the same as or functionally equivalent to a second amino acid in said identified portion;

(e) excising between one and all codons encoding the identified portion from the first nucleic acid molecule to produce an excised sequence the 3' codon of which encodes the second amino acid, an upstream codon which is the codon immediately preceding the excised sequence, and a downstream codon which is the codon immediately following the excised sequence;

(f) deleting zero or at least one codon from the 3' end of the second nucleic acid molecule to produce a third nucleic acid molecule the 3' codon of which is the codon encoding the first amino acid;

(g) inserting the third nucleic acid molecule between the upstream and downstream codons such that the 3' codon of the inserted third nucleic acid molecule encodes the first amino acid to produce a fourth nucleic acid molecule encoding the hybrid protein; and

(h) producing the hybrid protein from the fourth nucleic acid molecule.

73. A method of modifying a protein having a surface exposed loop of known sequence to produce a hybrid protein, which comprises:

(a) determining the mobility of amino acids in the loop to identify a portion thereof having the greatest mobility;

(b) providing a first nucleic acid molecule encoding at least the protein;

(c) providing a second nucleic acid molecule encoding a target amino acid sequence for eliciting an immune response;

(d) identifying a first amino acid proximate the carboxy terminus of the target sequence which is the same as or functionally equivalent to a second amino acid in said identified portion;

(e) deleting the codon encoding the first amino acid and all following codons from the 3' end of the second

nucleic acid molecule to produce a third nucleic acid molecule;

(f) identifying in the first nucleic acid molecule a downstream codon which is the codon encoding the second amino acid and an upstream codon which is the immediately adjacent codon at the 5′ end of the downstream codon;

(g) forming an insertion site in the first nucleic acid molecule such that the codon directly following the insertion site encodes the second amino acid;

(h) inserting the third nucleic acid molecule into the first nucleic acid molecule at the insertion site such that the 3′ codon of the inserted third nucleic acid molecule encodes the first amino acid, to produce a fourth nucleic acid molecule encoding the hybrid protein; and

(i) producing the hybrid protein from the fourth nucleic acid molecule.

74. The method of any one of claims 70 to 73, wherein said protein is a virus protein.

75. The method of claim 74, wherein the virus protein is a picornavirus protein.

76. The method of claim 75, wherein the deleting and inserting steps are effected by cassette mutagenesis.

77. The method of claim 76, wherein the first nucleic acid molecule comprises a first nucleic acid cassette including the upstream codon and the downstream codon; and further comprising:

(a) providing a second nucleic acid cassette having an upstream sequence, a downstream sequence, and a sequence between the upstream and the downstream sequence having the same sequence as the third nucleic acid molecule, such that the upstream sequence encodes the same amino acid sequence as the sequence of the first cassette from its 5′ end to the upstream codon inclusive and the downstream sequence encodes the same amino acid

sequence as the sequence of the first cassette from the downstream codon to its 3' end inclusive;

(b) excising the first nucleic acid cassette from the first nucleic acid molecule to form a fifth nucleic acid molecule;

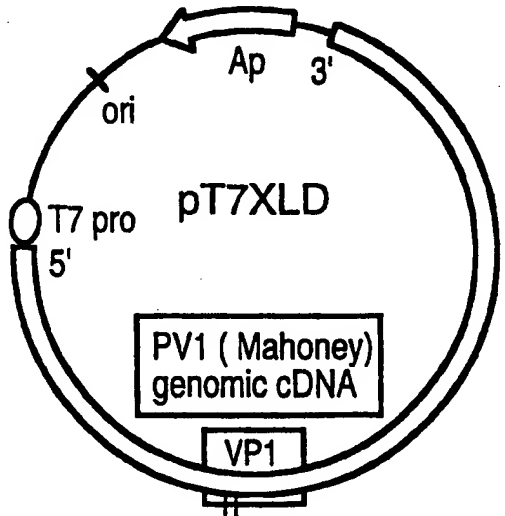
(c) inserting the second nucleic acid cassette into the second nucleic acid molecule to form a sixth nucleic acid molecule; and

(d) producing the hybrid protein from the sixth nucleic acid molecule.

78. The method of claim 76, wherein the immune response is a B cell or a T cell immune response.

79. The method of claim 78, wherein the hybrid protein is disposed on a hybrid picornavirus.

80. The method of claim 79, wherein the picornavirus protein is a polio protein and the hybrid picornavirus is a hybrid poliovirus.



Sal I Hind III

```
nt2753 G|TC GAC AAC CCA GCT TCC ACC ACC ATT AAG GAC A|AG CTT 2791
       CAG CT|G TTG GGT CGA AGG TGG TGG TTA TTC CTG TTC GA|A
aa1092 V   D   N   P   A   S   T   T   N   K   D   K   L   1104
```

Clone	Sequence encoded (Chlamydial sequences underlined)	Viable virus recovered/ strain designation
pT7XLD	NPASTT NKD	Yes PV1-XLD
pT7Ct2	VAGLEKDPVA	No
pT7Ct4	N PTTSDVAGLEKDPVA	No
pT7Ct5	N PTTSDVAGLEKDPKD	Yes PV1-Ct5
pT7Ct7	NPASTTSDVAGLEKDPVA	Yes PV1-Ct7
pT7Ct8	NPASTTSDVAGLEKDPKD	Yes PV1-Ct8

FIG.1.

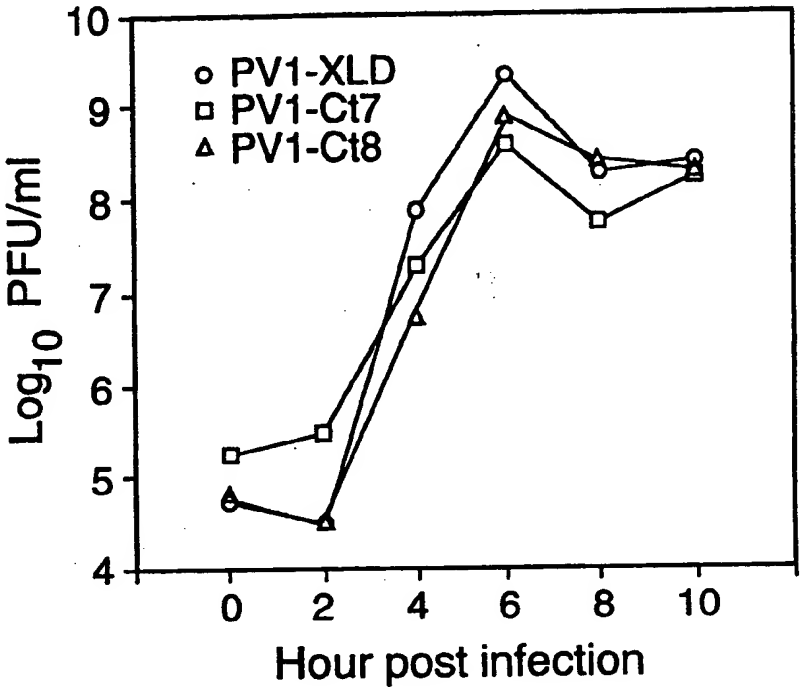

10
9
8
7
6
5
4
Log10 PFU/ml
PV1-XLD
PV1-Ct7
PV1-Ct8
0
2
4
6
8
10
Hour post infection

FIG.2.

Figure 3

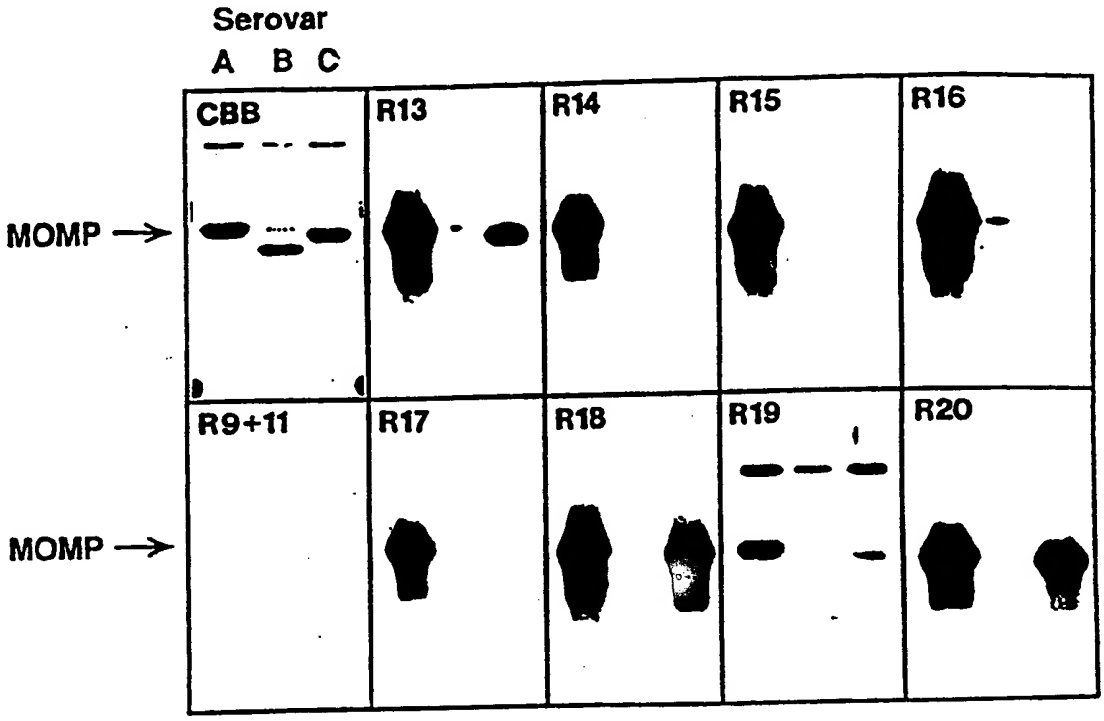
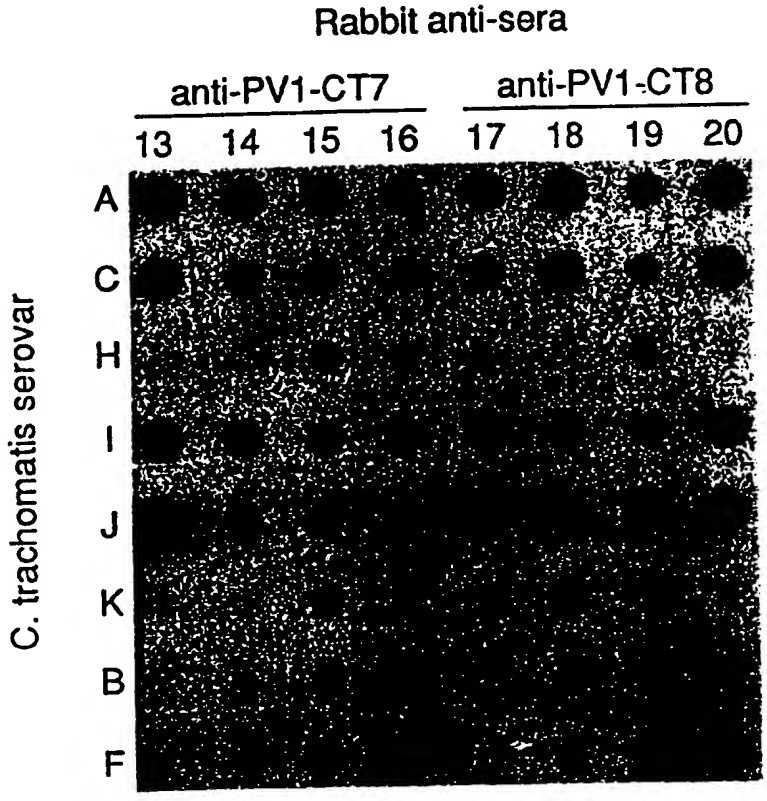


Figure 4


Rabbit anti-sera
anti-PV1-CT7
anti-PV1-CT8
13 14 15 16 17 18 19 20
C. trachomatis serovar
A
C
H
I
J
K
B
F

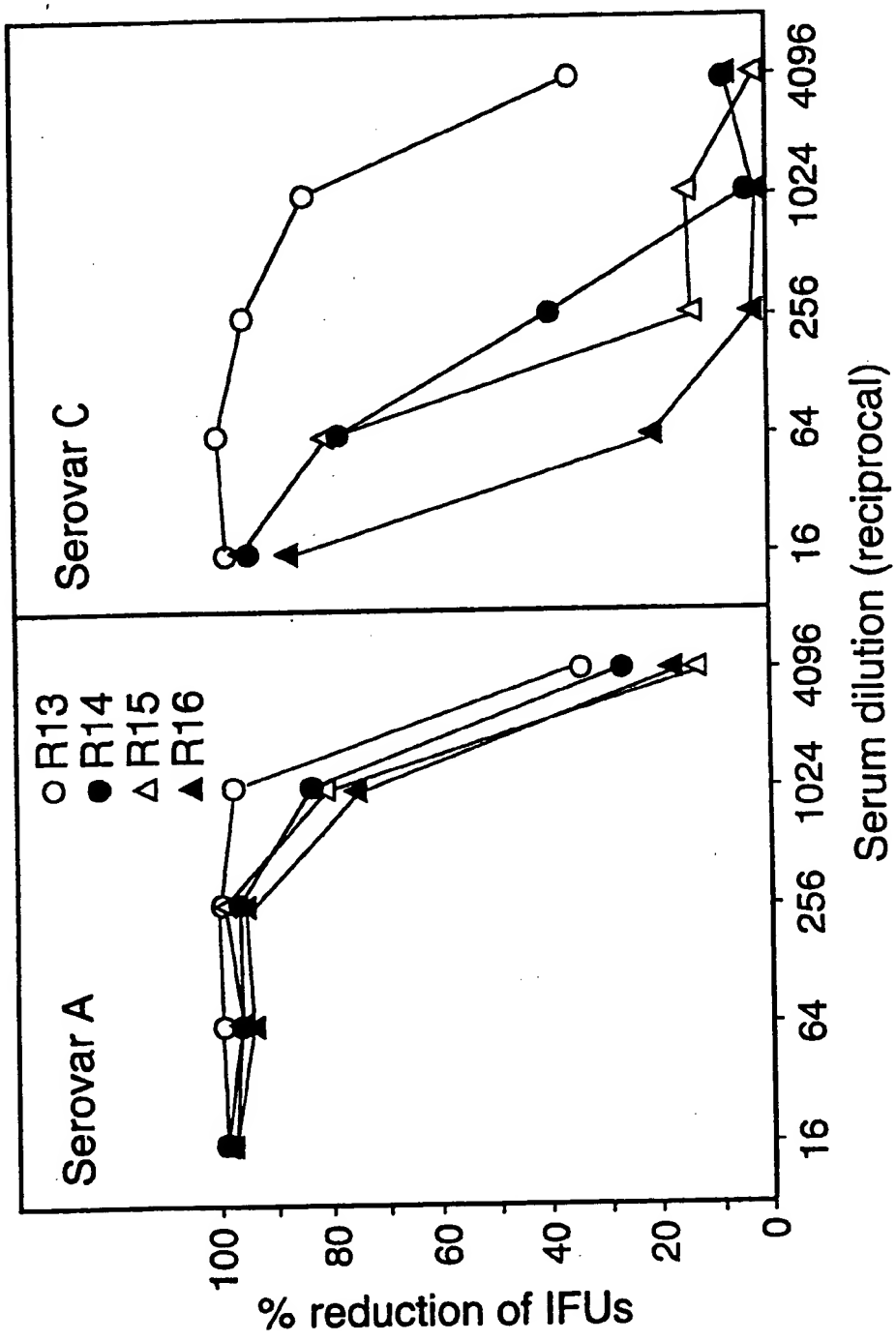
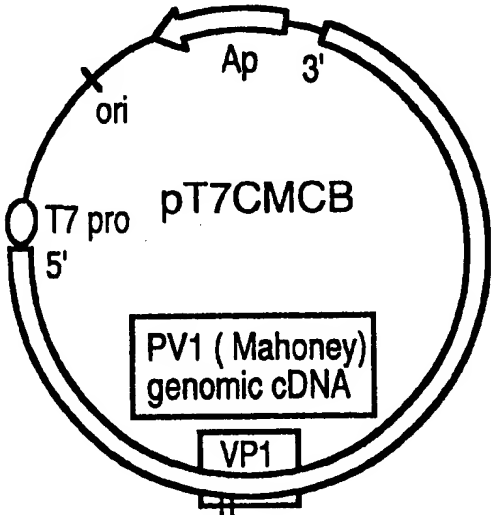

Serovar C
Serovar A
○R13
●R14
△R15
▲R16
% reduction of IFUs
100
80
60
40
20
0
16
64
256
1024
4096
Serum dilution (reciprocal)

FIG.5.

SUBSTITUTE SHEET



Sal I Hind III

```
nt2753 G|TC GAC AAC CCA GCT TCC ACC ACC ATT AAG GAC A|AG CTT 2791
       CAG CT|G TTG GGT CGA AGG TGG TGG TTA TTC CTG TTC GA|A
aa1094 V   D   N   P   A   S   T   T   N   K   D   K   L   1102
```

Clone	Sequence encoded (Chlamydial sequences underlined)	Viable virus recovered/ strain designation
pT7XLD	NPASTT NKD	Yes PV1-XLD
pT7CtIVA1	NPASTTLNPTIAGAGDVKD	Yes PV1-CtIVA
pT7CtIVB4	NPASTTLNPTIAGAGD D	Yes PV1-CtIVB
pT7CtIVC9	NPASTTLNPTIAGA D	Yes PV1-CtIVC

FIG.6.

Figure 7

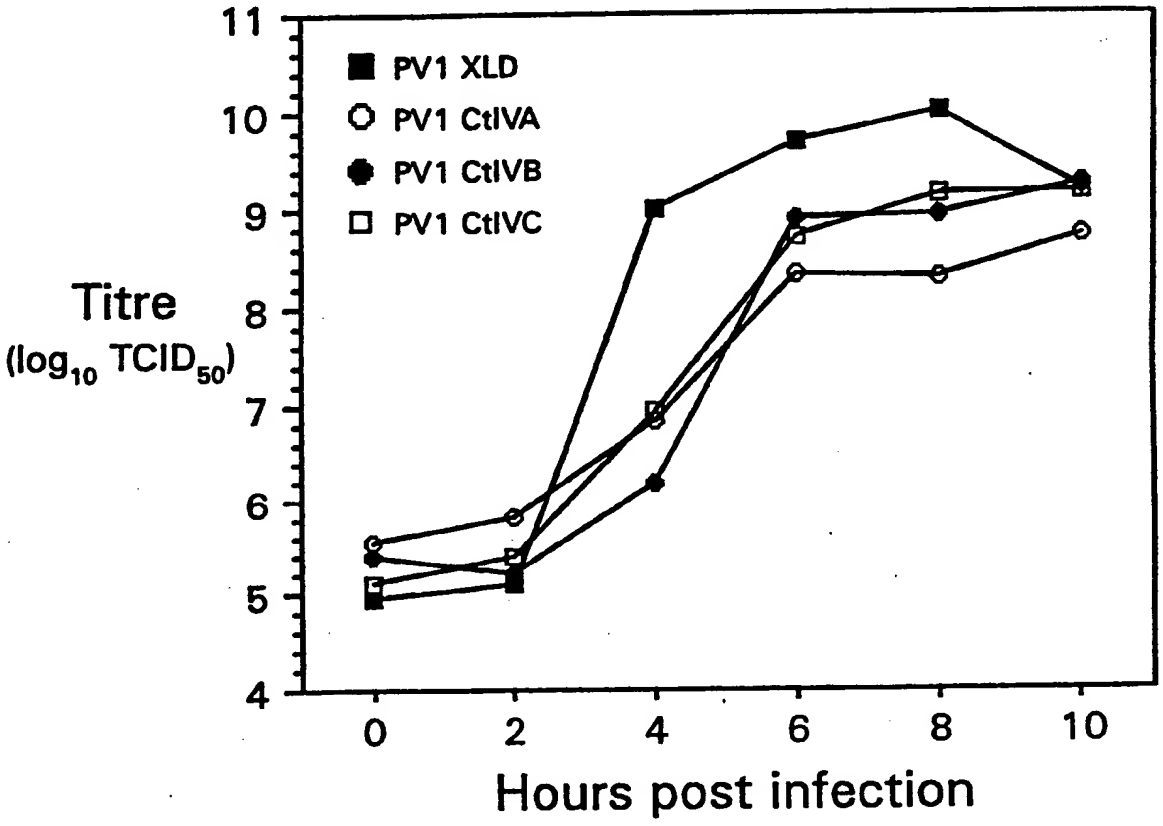
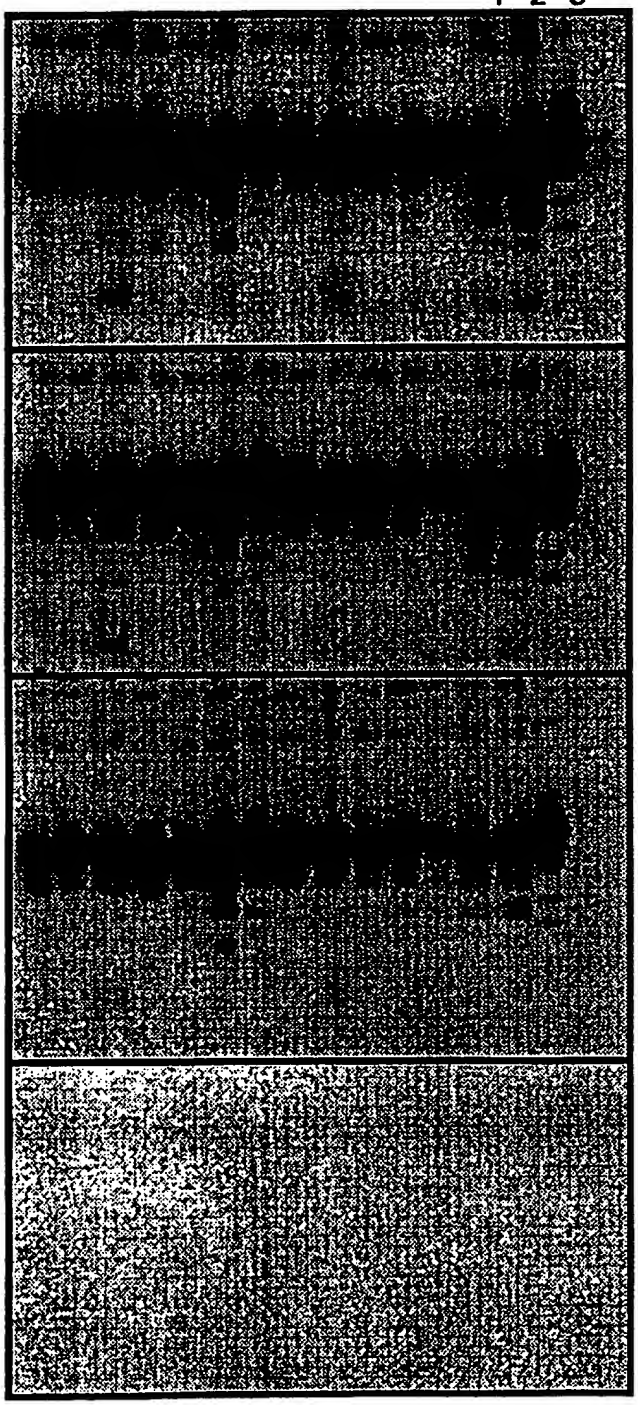

PV1 XLD
PV1 CtlVA
PV1 CtlVB
PV1 CtlVC
Titre
(log10 TCID50)
11
10
9
8
7
6
5
4
0
2
4
6
8
10
Hours post infection

Figure 8

***Chlamydia trachomatis* serovar**

B BaD E F G A C H I J K L_1 L_2 L_3 GPIC



(A) anti-PV1CtIVA

(pooled rabbit sera #s 24,25,26)

(B) anti-PV1CtIVB

(pooled rabbit sera #s 27,28,29)

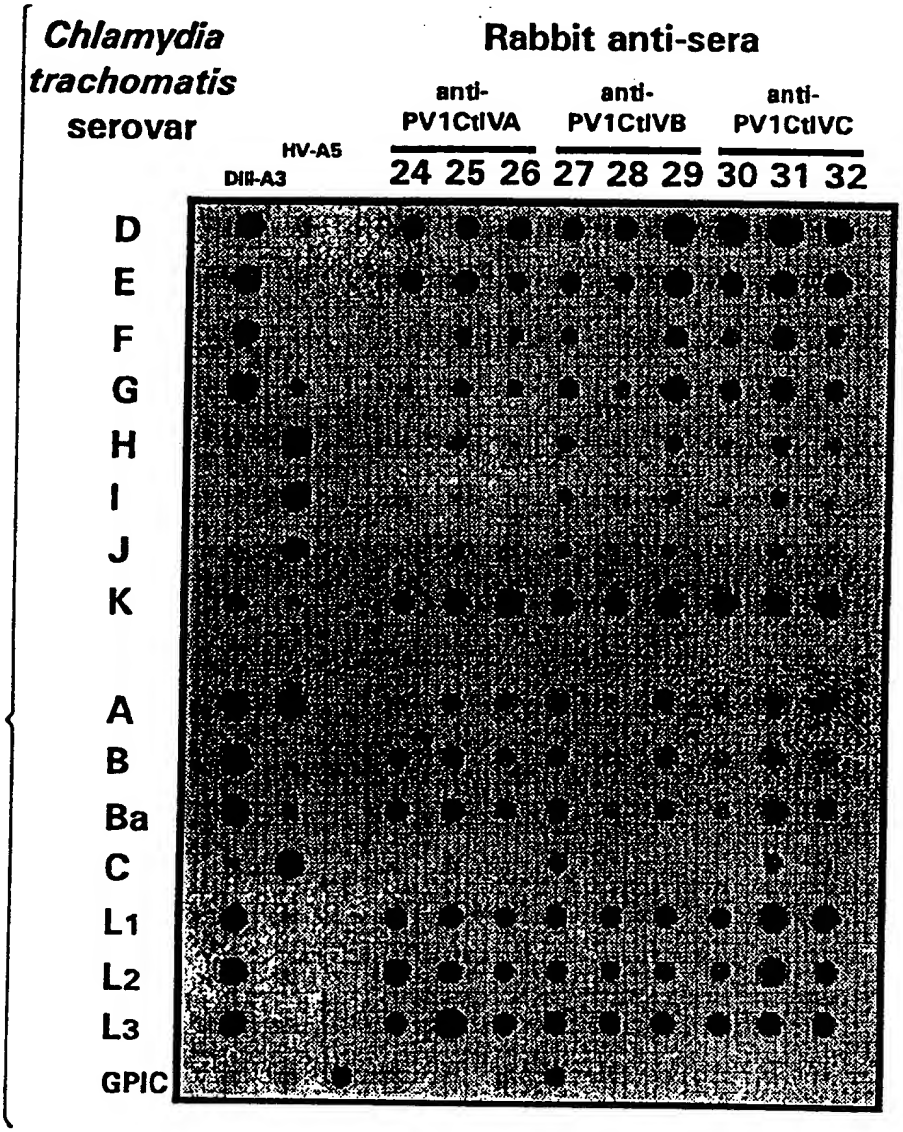
(C) anti-PV1CtIVC

(pooled rabbit sera #s 30,31,32)

(D) anti-PV1-XLD

(pooled rabbit sera #s 21,22,23)

Figure 9


Chlamydia trachomatis serovar
Rabbit anti-sera
anti-PV1CtlVA
anti-PV1CtlVB
anti-PV1CtlVC
HV-A5
DIII-A3
24 25 26 27 28 29 30 31 32
D
E
F
G
H
I
J
K
A
B
Ba
C
L1
L2
L3
GPIC

C. trachomatis Neutralizing Activity Of
Rabbit Anti-Poliovirus/MOMP Hybrid Sera

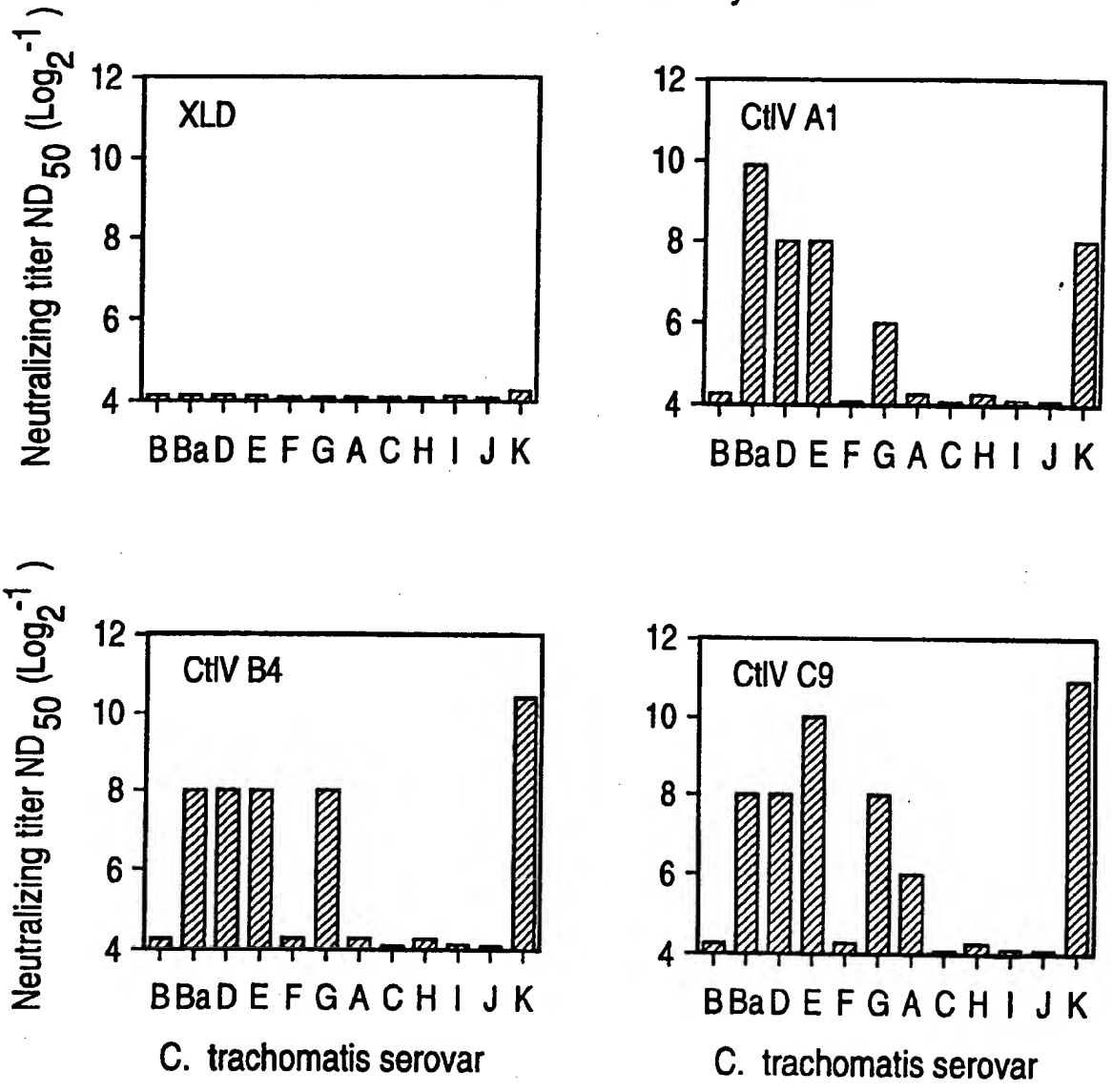


FIG.10.

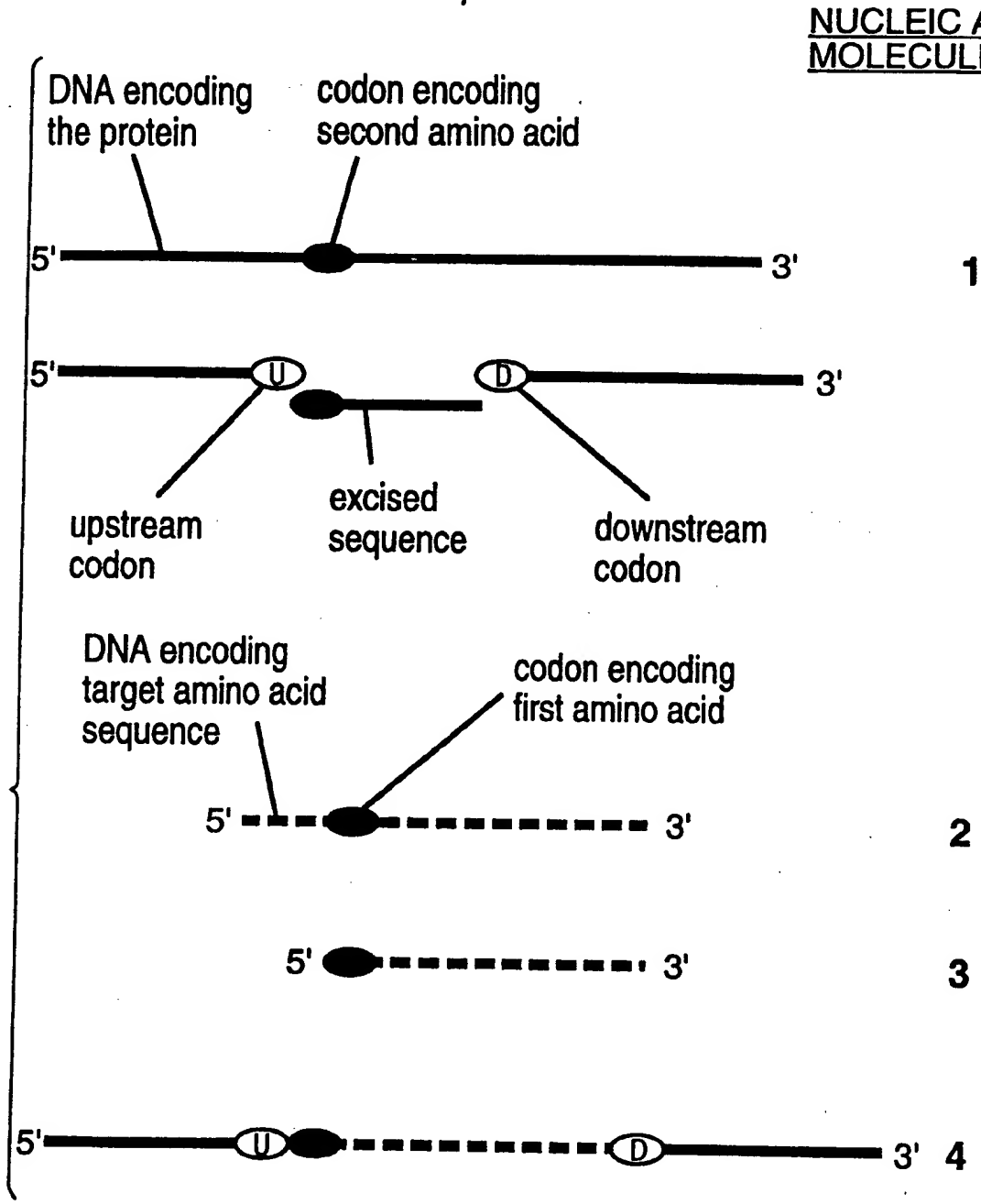

NUCLEIC ACID MOLECULE #
DNA encoding the protein
codon encoding second amino acid
5'
3'
1
5'
U
D
3'
upstream codon
excised sequence
downstream codon
DNA encoding target amino acid sequence
codon encoding first amino acid
5'
3'
2
5'
3'
3
5'
U
D
3'
4

FIG.11.

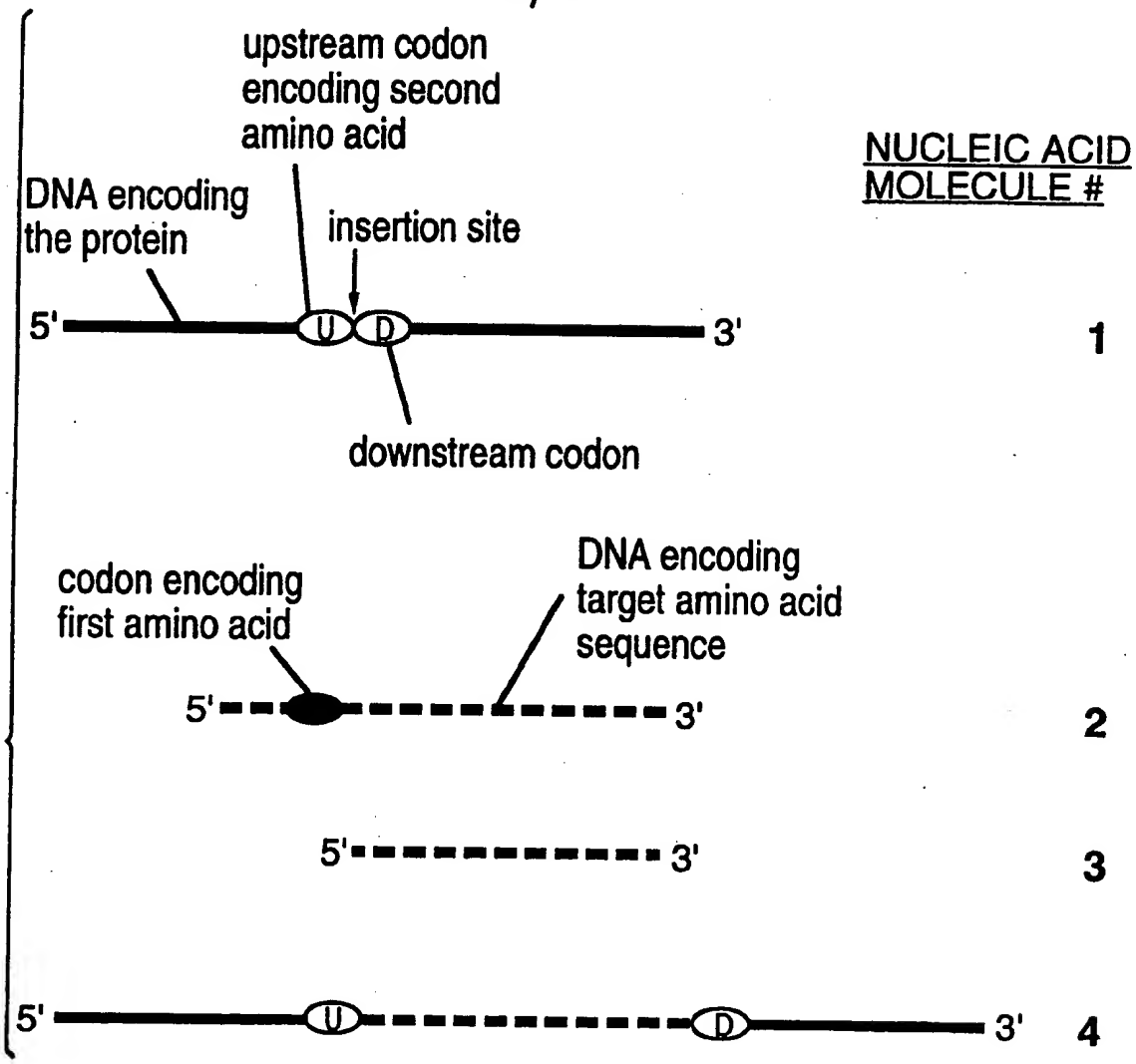

upstream codon
encoding second
amino acid
DNA encoding
the protein
insertion site
5'
U
D
3'
downstream codon
NUCLEIC ACID
MOLECULE #
1
codon encoding
first amino acid
DNA encoding
target amino acid
sequence
5'
3'
2
5'
3'
3
5'
U
D
3'
4

FIG.12.

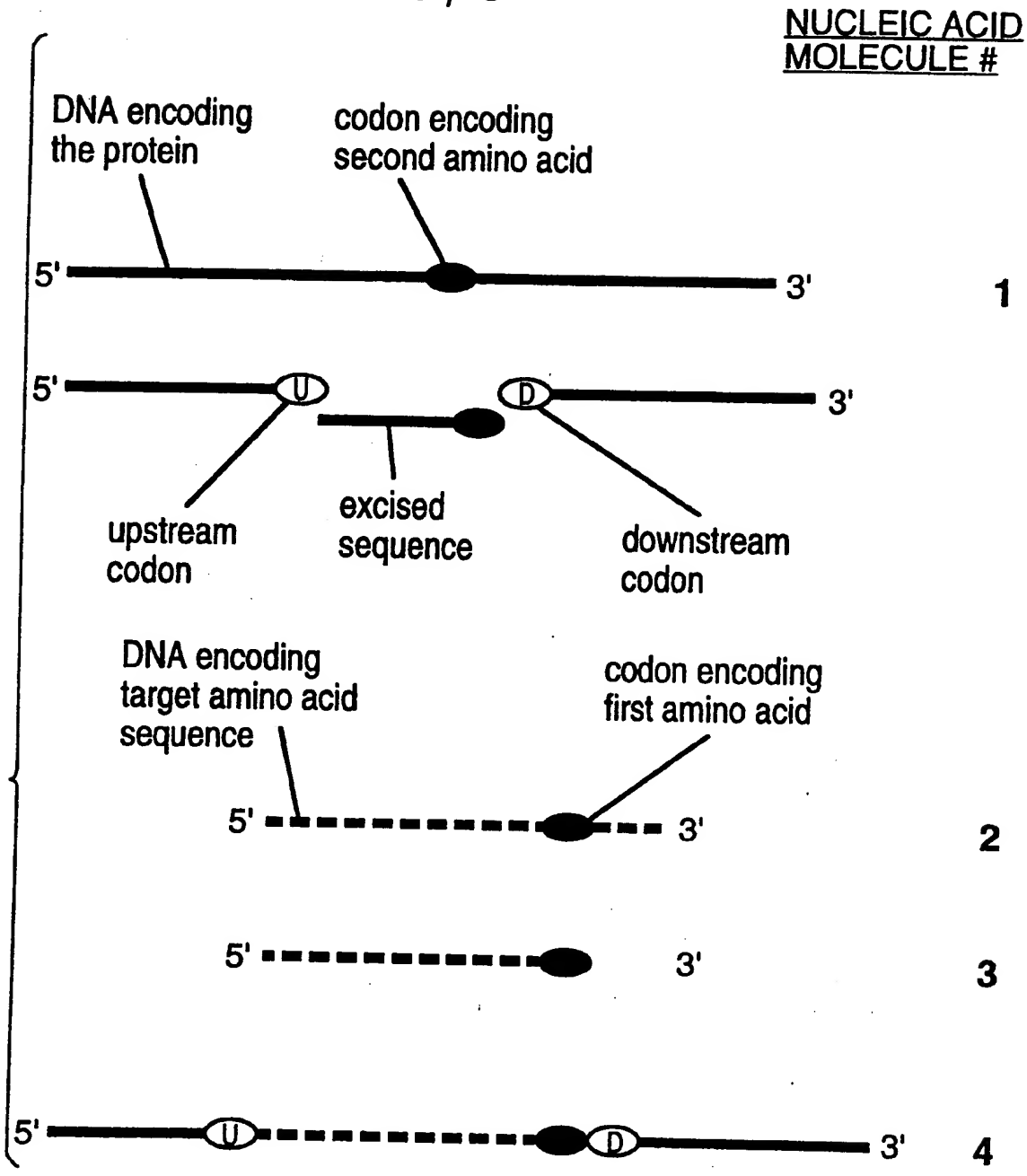

NUCLEIC ACID MOLECULE #
DNA encoding the protein
codon encoding second amino acid
5'
3'
1
5'
U
D
3'
upstream codon
excised sequence
downstream codon
DNA encoding target amino acid sequence
codon encoding first amino acid
5'
3'
2
5'
3'
3
5'
U
D
3'
4

FIG.13.

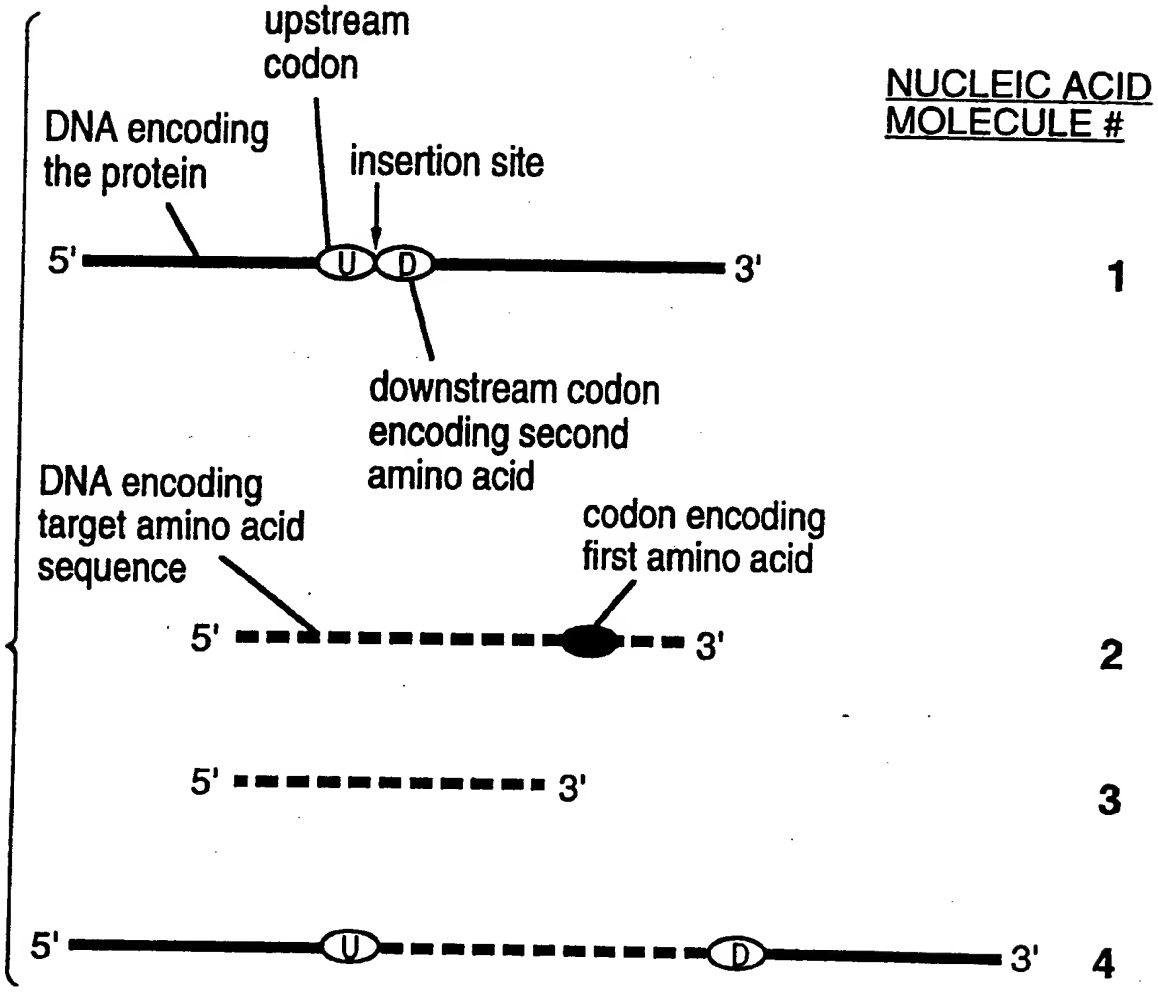

upstream codon
DNA encoding the protein
insertion site
5'
U
D
3'
downstream codon encoding second amino acid
DNA encoding target amino acid sequence
codon encoding first amino acid
5'
3'
5'
3'
5'
U
D
3'
NUCLEIC ACID MOLECULE #
1
2
3
4

FIG.14.

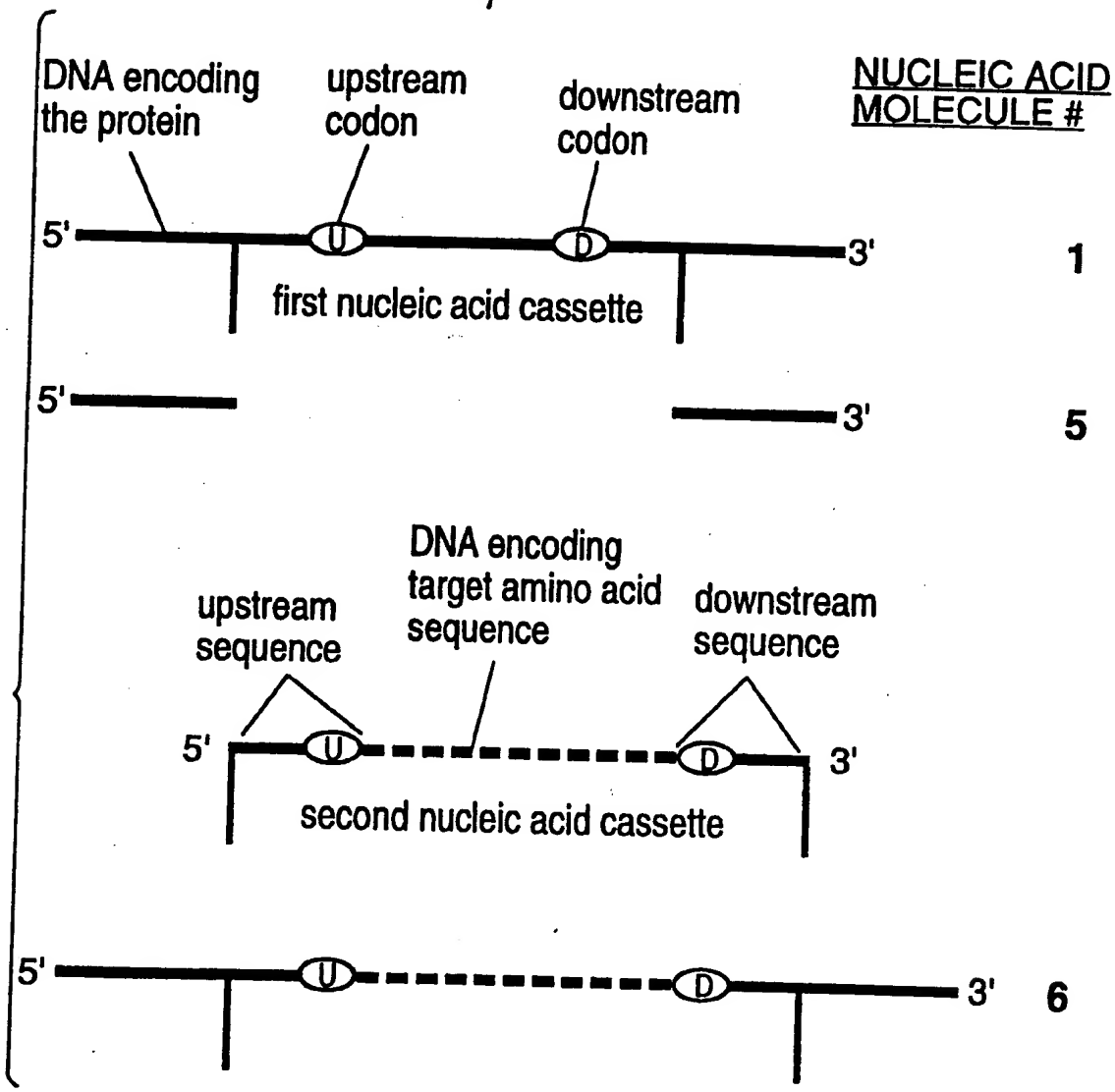

DNA encoding the protein
upstream codon
downstream codon
NUCLEIC ACID MOLECULE #
5'
U
D
3'
1
first nucleic acid cassette
5'
3'
5
DNA encoding target amino acid sequence
upstream sequence
downstream sequence
5'
U
D
3'
second nucleic acid cassette
5'
U
D
3'
6

FIG.15.

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